THE UNIVERSITY *of* EDINBURGH

Title	Investigation of the cardiac endothelin system post myocardial infarction in the rat
Author	Sherry, Lorcan Adrian
Qualification	PhD
Year	2001

Investigation of the cardiac endothelin system post myocardial infarction in the rat

Lorcan Adrian Sherry

PhD – The University of Edinburgh – 2000

Declaration

I hereby declare that the work described in this thesis was performed entirely by myself, except for procedures acknowledged in the text, and that it has not been accepted in any previous application for a degree.

Lorcan Sherry

Acknowledgements

Foremost I would like to thank my principal supervisor, Dr. Gillian Gray, whose continual support and advice throughout my PhD was very much appreciated. I would also like to thank Prof. David Webb for his continued advice over the past 3 years.

A special word of thanks must go to Dr. Pauline McEwan for her time and 'patience' in teaching me laboratory techniques. I also acknowledge the kind help of Mr. Neil Johnston, Ms Isa Ramsey, Mrs Jean Hunter, Mr. Mark Patrizio, and Mr. David O'Regan.

I would finally like to thank my family and friends for their support and encouagement throughout my university career.

Abstract

Survivors of acute myocardial infarction (MI) have a high risk of developing chronic heart failure (CHF). In human CHF there is evidence that plasma levels of the potent vasoconstrictor peptide, endothelin (ET)-1 and its precursor big ET-1 are increased, correlate positively with disease severity, and may be important predictors of outcome. The aims of this thesis were to investigate the heart as a source of ET-1 synthesis and to investigate the role of the cardiac ET-1 system during scar formation in the early stage post-MI and also during progression of CHF in a rat model. MI in the rat was induced by surgical ligation of the left anterior descending coronary artery. The myocardial ET system was investigated using immunohistochemical and *in situ* hybridisation techniques.

In the normal heart, immunoreactive ET-1 was identified in the vascular endothelium and myoendothelial cells in the myocardium. Staining for ET-1 was intense and uniform in cardiomyocytes throughout the myocardium. *In situ* hybridisation confirmed that sites of preproET-1 mRNA expression coincided with sites of immunoreactive ET-1. These results indicate that the myocardium has the capacity to endogenously produce ET-1 in both myoendothelial cells and cardiomyocytes.

Investigation of ET-1 immunoreactivity during scar formation 2, 7 and 14 days post-MI indicated an early transient increase in ET-1 in the infarct, which peaks at 7 days. At this time point, ET-1 could be localised to proliferating fibroblasts, infiltrating inflammatory cells and in the endothelia of newly forming vessels, suggesting a role for ET-1 in scar formation early post-MI.

Though increased ET-1 staining could be localised at a cellular level in the developing infarct, further studies were needed to identify whether ET-1 was beneficial or detrimental during scar formation. Therefore, the effect on early stage scar formation of an $ET_A/_B$ receptor antagonist (A-182086) fed to rats immediately after coronary artery ligation surgery and for the duration (2, 7 or 14 days) of the study was investigated. In rats treated with antagonist, viable myocardial content of

the infarcted region was significantly greater at both 7 and 14 days post-MI compared to untreated rats. The salvaged myocardium also demonstrated a vascular supply and ET-1 immunoreactivity similar to that observed in normal viable myocardium from sham-operated rats.

In rat hearts 5 and 12 weeks post-MI, the area of infarction induced by coronary artery ligation had formed a fibrosed scar. ET-1 staining was located uniformly in all non-infarcted areas of the heart, but only in the few remaining viable myocytes in the infarcted region. PreproET-1 mRNA expression matched ET-1 immunohistochemical visualisation in all areas of the heart. ET_A receptor expression was similar in the non-infarcted areas of post-MI rat hearts compared to that in areas from age-matched sham-operated rat hearts. However, ET_B receptor expression and ET_B immunoreactivity were increased in the non-infarcted areas of rat hearts at 12 weeks but not 5 weeks post MI and co-localised with an increase in the transcriptional growth factor ERK.

These results suggest that the heart has the capacity to endogenously produce ET-1 and that changes in the ET-1 system occur at different stages post-MI. Furthermore, these changes may be detrimental to acute myocardial cell survival post-MI, and in the later stages post-MI during progressive remodelling of the non-infarcted myocardium.

Publications

Abstracts and presentations:

Sherry L, McEwan PE, Webb DJ, Gray GA. (1997) 'Effect of chronic heart failure on endothelin-1 distribution in the rat heart'. Poster presentation at the British Society for Cardiovascular Research, Bristol, September, 1997.

McEwan PE, Sherry L, Webb DJ, Gray GA. (1998) 'Localisation of myocardial prepro ET-1 mRNA in a rat model of CHF'. *J. Mol. Cell Cardiol.*, **30**, A396. Moderated poster presentation at the International Society for Heart Research, Rhodes, Greece, May, 1998.

Sherry L, McEwan PE, Webb DJ, Gray GA. (1998) 'Characterisation of ET-1, TGF-β and collagen levels in early stage scar formation after MI in the rat'. Poster presentation at the Scottish Cardiovascular Forum, October, 1998 and oral communication at the Scottish Society for Experimental Medicine, Edinburgh November, 1998.

Sherry L, McEwan PE, Webb DJ, Gray GA (1999) 'Selective upregulation of left ventricular ET_B receptor expression post-myocardial infarction'. Poster presentation at the First Oslo International Conference on Molecular Cardiology and Myocardial Function, May, 1999.

Sherry L, McEwan PE, Webb DJ, Gray GA (1999) 'Early endothelin receptor antagonist intervention post-myocardial infarction improves cardiomyocyte survival in the infarct zone and reduces the mass of the non-infarcted myocardium'. Oral communication at the Sixth International Conference on Endothelin, Montreal, Canada, October, 1999. Young Investigator Award for best oral presentation.

Full Papers:

McEwan PE, Gray GA, Sherry L, Webb DJ, Kenyon, CJ. (1998) Differential effect of Angiotensin II on cardiac cell proliferation and intramyocardial perivascular fibrosis *in vivo*. *Circulation*, **98**, 2765-2773.

McEwan PE, Sherry L, Kenyon, CJ, Webb DJ, Gray GA. (2000) Regulation of the myocardial endothelin system by angiotensin II and losartan. *J. Cardiovasc. Pharmacol.*, In press.

Papers in preparation:

McEwan PE, Sherry L, Patrizio M., Webb DJ, Gray GA. Endothelin B receptors are upregulated post myocardial infarction in the rat and co-localise with early growth genes in the hypertrophied non-infarcted left ventricle.

Sherry L, McEwan PE, O'Regan D., Webb DJ, Gray GA. The effect of early administration of non-selective endothelin receptor antagonism on scar formation post myocardial infarction in the rat.

Sherry L, McEwan PE, Webb DJ, Gray GA. Specific cellular localisation of TGF-β1 in early stage scar formation after MI in the rat.

Magazine Articles:

Meeting report: 1st Oslo Conference on Molecular Cardiology and Myocardial Function. Produced for 'The Bulletin', The publication of the British Society for Cardiovascular Research.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AV	Atrio-ventricular
ACE	Angiotensin converting enzyme
AT	Angiotensin receptor
Ang II	Angiotensin II
ANP	Atrial natriuretic peptide
AP	Alkaline phosphatase
AVP	Arginine vasopressin
BID	Twice daily
bFGF	Basic fibroblast growth factor
BK	Bradykinin
BNP	Brain natriuretic peptide
BP	Blood pressure
Ca^{2+}	Calcium ion
cAMP	cyclic 3',5' –adenosine monophoshpate
CAD	Coronary artery disease
CAL	Coronary artery ligation
cGMP	cyclic 3',5' –guanosine monophoshpate
CHF	Chronic heart failure
CNS	Central nervous system
CO	Cardiac output
DAB	3,3'-diaminobenzidine tetrachloride
DAG	Diacyl glycerol
DIG	Digoxygenin
DNA	Deoxyribonucleic acid
dP/dt	Change in ventricular pressure over time (a measure of contractility)
EC_{50}	Dose of drug which produces 50% of its maximum response
ECE	Endothelin converting enzyme
EGF	Endothelial growth factor
EDTA	Ethylenediaminetetraacetic acid

ERK	Extracellular regulated protein kinase
ET-1	Endothelin-1
ET_A	Endothelin-A receptor
ET_B	Endothelin-B receptor
GSL	Griffonia simplicifolia lectin
H&E	Haematoxylin and eosin
INF	Infarct
IP	Intraperitoneal
IP_3	Inositol-3-phosphate
K_d	the concentration of drug which, at equilibrium, occupies 50% of the receptors (units M/L)
LAD	Left anterior descending
LVEDP	Left ventricular-end diastolic pressure
LV	Left ventricle
MAP	Mean arterial pressure
MAPK	Mitogen activated protein kinase
MHC	Myosin heavy chain
MI	Myocardial infarction
mRNA	messenger Ribonucleic acid
NBT/BCIP	Nitroblue tetrazolium chloride/5-bromo-4-chloride-3-iodolyl phoshpate
NO	Nitric oxide
NOS	Nitric oxide synthase
NYHA	New York Heart Association
PDGF	Platelet derived growth factor
PBS	Phosphate buffered saline
RAAS	Renin-angiotensin-aldosterone system
RV	Right ventricle
SR	Sarcoplasmic reticulum
SRTX	Sarafotoxin
NF	Nuclear factor
PBS	Phosphate buffered saline

PLC	Phospholipase C
PKA	Protein kinase A
PKC	Protein kinase C
TBS	Tris buffered saline
TGF-β_1	Transforming growth factor beta-1
TNFα	Tumour necrosis factor-α
TUNEL	*In situ* nick end-labelling
VSMC	Vascular smooth muscle cell

Chapter 1

Introduction

1.1 Anatomy and physiology of the normal working heart

The heart and blood vessels comprise the circulation system whose basic function is to pump blood and provide adequate flow to the various organs in the body, thereby maintaining oxygen and substrates while removing metabolites and other materials.

The wall of the heart is split into three layers, the inner endocardium which consists of simple squamous epithelium and a layer of connective tissue; the middle layer, myocardium, which contains a layer of cardiac muscle, and finally the outer pericardium, a two layered serous membrane enclosing the whole heart (reviewed by MacKenna & Calander, 1997). The myocardium consists of many different groups of cells including 'working' myocardial cells, specialised 'conducting' cells, and vascular and connective tissue. Working cells predominantly consist of cardiac myocytes, which occupy 75% of the myocardial space. The individual ventricular myocytes that account for more than half of the heart's weight are roughly cylindrical in shape and measure about 10-25μm in diameter and about 50-100μm in length (Moody *et al.*, 1990). Those in the atrium are smaller, being less than 10μm in diameter and about 20μm in length. Although there are many different cell types in the heart, it is the ventricular myocytes that, by their contraction, propel blood around the heart.

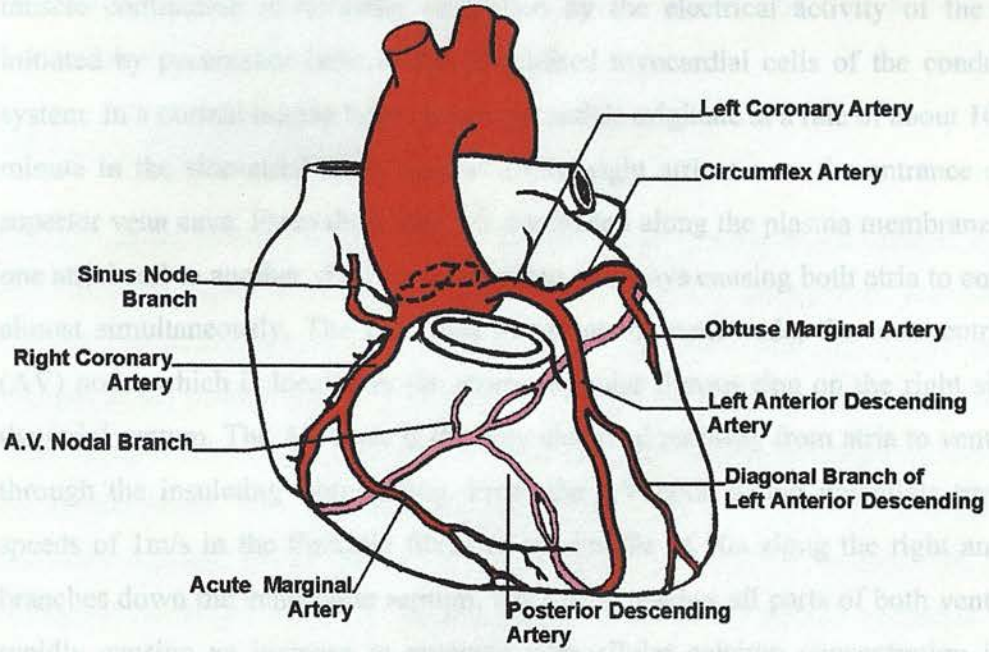
A collagen network, composed largely of type I and III fibrillar collagens, is found in the extracellular space of the myocardium (Weber *et al.*, 1994). Collagen fibres course through the myocardium surrounding and grouping muscle fibres into bundles. Collagen also surrounds individual myocytes connecting them to one another and to their neighbouring capillaries. Support and alignment of myocytes, blood vessels and lymphatic vessels relative to one another preserve myocardial thickness and architecture. The network also prevents cardiac myocyte slippage and myocytes from overstretching, while being the basis for diastolic myocardial stiffness and adding tensile strength to the myocardium to prevent rupture. Overall, the collagen network preserves architecture and stiffness of the myocardium, and the geometry of ventricular chambers would be weakened without it.

Other components of cardiac connective tissue include fibroblasts, which are mesenchymal cells that potentially produce components of the extracellular matrix including collagen and fibronectin. The matrix metalloproteases include various enzymes (such as collagenase) that break down all the types of collagen as well as other components of the extracellular matrix including laminin, fibronectin and other glycoproteins. The balance between the synthesis of extracellular matrix collagen by various growth factors and neurohormonal peptides versus degradation by the metalloproteases has important implications for the mechanical properties and hence the function of the myocardium.

Vascular smooth muscle cells are found in epicardial and intramyocardial coronary arteries and are different in shape and function from striated myocardial cells. These cells are adapted to much slower rates of contraction and relaxation and to the maintenance of sustained tonic contractions. Their major function directly on the heart is to maintain coronary vascular tone while indirectly, through regulation of peripheral vascular resistance, controlling the resistance against which the heart pumps out blood. Vascular smooth muscle cells, like endothelial cells and fibroblasts, are capable of re-entering the cell cycle and can therefore undergo mitosis and hyperplastic growth. On the other hand, adult cardiomyocytes are thought to be terminally differentiated and, therefore, do not proliferate (Weber & Brilla, 1991).

Because work production and energy requirement of the heart vary so much from rest to exercise, there must be some system of variable oxygen delivery to the myocardium. Blood reaches the cardiac myocytes via the coronary circulation. Two major coronary arteries run from the base of the aorta to the left and right ventricles, respectively, before giving off branches that run down the surface of the heart toward the apex. The major branch of the left coronary artery, which supplies the left ventricle, is the left anterior descending coronary artery (*Figure 1.1*).

Figure 1.1 Diagram of heart showing the major coronary vessels that supply blood to the myocardium. Modified from Sobotta, Atlas of the anatomy of the body, 21st edition.



The control of the myocardial oxygen supply lies in the coronary arterioles, which keep branching until the very small, thinned-walled capillaries are formed. It is here that transfer of oxygen from oxygenated arterial blood to the myocardial tissues occurs. Endothelial cells in the atrial and ventricular myocardium form the lining of both coronary vessels and the fine coronary capillary network that runs thoughout the myocardium, these endothelial cells being termed myoendothelial cells. At the level of the coronary microvasculature, there is close contact between endothelial cells and cardiac myocytes throughout the heart, with no cardiac myocyte being more than 2-3μm from a coronary vasculature endothelial cell.

1.1.1 The Cardiac Cycle

The interrelationship amongst electrical, mechanical and valvular events during one complete heartbeat is referred to as the *cardiac cycle*. The rhythmicity of cardiac muscle contraction is normally controlled by the electrical activity of the heart initiated by pacemaker cells in the specialised myocardial cells of the conducting system. In a normal human heart, action potentials originate at a rate of about 100 per minute in the sino-atrial node, located in the right atrium near the entrance of the superior vena cava. From there they are conducted along the plasma membrane from one atrial cell to another via three conducting pathways causing both atria to contract almost simultaneously. The pathways merge at the next node, the atrioventricular (AV) node, which is located in the atrioventricular fibrous ring on the right side of the atrial septum. The AV node is the only electrical pathway from atria to ventricles through the insulating fibrous ring. From the AV node action potentials travel at speeds of 1m/s in the Purkinje fibres of the bundle of His along the right and left branches down the ventricular septum. Excitation reaches all parts of both ventricles rapidly causing an increase in myocyte intracellular calcium concentration in the vicinity of the sarcoplasmic reticulum (SR). This leads to the release of large amounts of calcium from the SR that reacts with the contractile proteins of the myofibrils causing contraction.

1.1.2 Neurohormonal regulation in the normal heart.

1.1.2.1 Control of heart rate

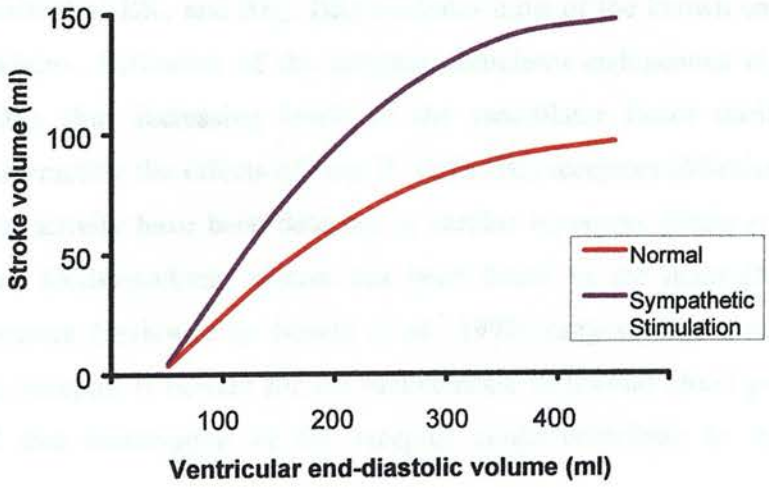
The heart rate of a resting adult human is 50-100 beats/minute while in small mammals it is faster (e.g. rat, 400-500 beats/minute). Autonomic control of the heart is split into two components, the parasympathetic nervous system, which decreases heart rate and the sympathetic nervous system, which increases heart rate. The parasympathetic fibres to the heart are carried by the vagus nerve. Parasympathetic nerve terminals act by releasing the neurotransmitter acetylcholine, which binds to muscarinic receptors, leading to a fall in intracellular cyclic 3',5'-adenosine monophosphate (cAMP) and reduced activation of sodium channels involved in

propagation of the depolarising inward pacemaker current. Unlike the parasympathetic fibres, sympathetic fibres richly innervate ventricular muscle as well as the atria and electrical system. The sympathetic nerve terminals act by releasing the neurotransmitter noradrenaline. Noradrenaline binds to β_1-adrenoreceptors on the pacemaker cell membrane increasing intracellular cAMP, and over the course of several beats this leads to an increase in firing rate.

1.1.2.2 Control of stroke volume

Changes in the force of contraction can be produced by a variety of factors, but two are dominant under most physiological conditions: (1) changes in end-diastolic volume, that is, the volume of blood in the ventricles just before contraction; and (2) changes in the magnitude of sympathetic nervous system input to the ventricles. The relationship between stroke volume and end-diastolic volume is known as *Starling's law of the heart*. This law states that an increase in venous return automatically forces an increase in cardiac output by increasing end-diastolic volume and hence stroke volume (*Figure 1.2*). As normal cardiac muscle is stretched, contractile force continuously rises to reach a maximum, but further stretch causes no decline in contractile force.

Figure 1.2 The Frank-Starling curve under normal conditions (red), and under stimulation by sympathetic nerves to the heart (purple).



Stimulation by the sympathetic nerves (via noradrenaline) increases ventricular contractility. The Frank-Starling mechanism still applies, but during nerve stimulation the stroke volume is greater at any given end-diastolic ventricular volume. Therefore, increased contractility leads to a more complete ejection of the end-diastolic ventricular volume.

1.1.3 Autocrine and paracrine modulation of myocardial function

The contribution of endogenous angiotensin (Ang) II to the physiological regulation of cardiac contractile function remains uncertain, though a locally active renin-angiotensin system is now known to be present within the human and animal heart (reviewed by Danser *et al.*, 1999) and is involved in disease states such as left ventricular hypertrophy and congestive heart failure (Lijnen. & Petrov, 1999; Kim & Iwao, 2000). Ang II is formed from Ang I by the activity of two major enzymes, angiotensin converting enzyme (ACE) and heart chymase (Urata *et al.*, 1991). ACE present in endothelial cells and cardiac myocytes in the heart (Hokimoto *et al.*, 1996; Paul *et al.*, 1996) also has kininase activity, thus modulating local levels of bradykinin as well as Ang I and II. Sacrolemmal receptors for Ang II are present on cardiac myocytes (Sadoshima & Izumo, 1993). Ang II is a direct positive inotrope and also facilitates sympathetic nervous influences on the heart (Paul *et al.*, 1992).

The biological effects of bradykinin (BK) are mediated through specific receptors, classified as BK_1 and BK_2. BK_2 mediates most of the known cardiovascular effects of kinins. Activation of the receptor stimulates endogenous nitric oxide synthase (NOS), thus increasing levels of the vasodilator factor nitric oxide (NO) and counteracting the effects of Ang II. Both BK_2 receptors (Minshall *et al.*, 1995) and NOS activity have been detected in cardiac myocytes (Stein *et al.*, 1996), and an intact kallikrein/kinin system has been found in the heart (Nolly *et al.*, 1997). Literature (reviewed by Nambi *et al.*, 1992) suggests that a normally functioning BK_2 receptor is needed for the maintenance of normal blood pressure homeostasis and that inactivation of the receptor could contribute to the development of

hypertension by leaving the activity of endogenous vasoconstrictor agents unopposed.

The family of natriuretic peptides which include atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) are both expressed in the heart, with alterations occurring during development and in disease states (reviewed by Forssmann *et al.*, 1998). Atrial cells store and release ANP, which has powerful effects on the kidney, stimulating sodium and water excretion, and the vasculature mediating vasorelaxation. Release of ANP occurs in response to stretching of the atria by increased central venous pressure, signalling volume overload of the circulation. Unlike ANP, produced by the atria, BNP is mainly synthesised and released into the circulation by the left ventricle and is therefore influenced by stimuli involving this cardiac chamber, such as an increase in arterial pressure, left ventricular hypertrophy and dilation. Both peptides exert their effects by combining with membrane receptors on vascular endothelial cells leading to generation of cyclic 3',5'guanosine monophoshpate (cGMP) and vasorelaxation, similar to the pathway activated by NO. Plasma BNP levels are very low in healthy subjects, and respond modestly, but significantly to physiological stimuli such as changes in posture or sodium intake (La Villa *et al.,* 1995).

Effects of endothelin-1 on the heart will be discussed in Section 1.2.5.2.

1.2 The Endothelin Family

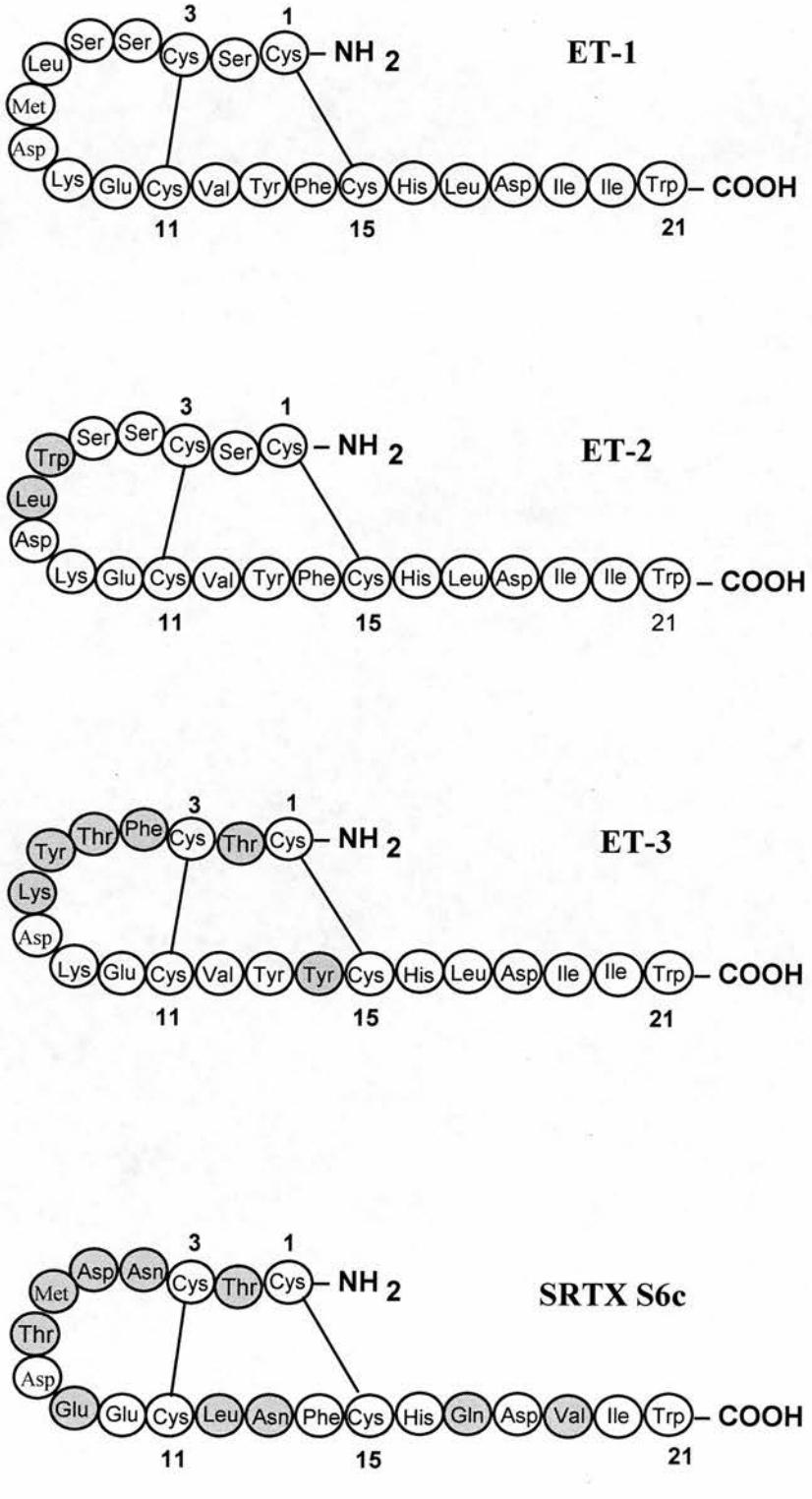
1.2.1 Discovery and Background

Past experiments on isolated arteries and veins have shown that either hypoxia (Rubanyi & Vanhoutte, 1985) or anoxia (Demay, & Vanhoutte 1983; Detar & Bohr, 1972) could induce endothelium-dependent contractions. In 1985, Hickey *et al.* demonstrated that a culture medium of bovine aortic endothelial cells triggered a slowly developing and long lasting contraction of isolated pig arteries, which could not be attributed to any known vasoconstrictor mediators and was peptidergic in nature. A subsequent study confirmed this observation (Gillespie *et al.*, 1986).

In 1988, Yanagisawa *et al.* reported that this endothelium-derived factor, one of the most potent constrictors described to date, was a 21 amino acid peptide, which they termed endothelin (ET; (Yanagisawa *et al.*, 1988)). The EC_{50} value of the vasoconstrictor activity of ET-1 in porcine coronary artery measured by Yanagisawa *et al.* (1988) was $3x10^{-10}$M. This value is about 100-fold more potent than that of Ang II, which was the most potent vasoconstrictor peptide until that time.

Subsequent studies have shown that ET-1 isolated from endothelial cells is one of a family of isopeptides (*Figure 1.3*), all of which are formed through a three-step processing pathway from their respective precursor peptides (*Figure 1.4)* that share high sequence homology, but are encoded by distinct genes. Four isopeptides (ET-1, $ET\text{-}1_{1\text{-}31}$, ET-2 and ET-3) have now been identified in the ET family; predicted by three separate genes, of which ET-1 is the most potent in the cardiovascular system. ET-1 was primarily identified in endothelial cells, though later studies have also identified ET-1 in rat (Giaid *et al.*, 1991), human (Giaid *et al.*, 1995) and porcine (Tonnessen *et al.*, 1995) cardiomyocytes and *in vitro* studies have established stimulated production of ET-1 in smooth muscle cells under pathological conditions (Hanehira *et al.*, 1997).

Figure 1.3 Structures of the three human endothelin peptides and Sarafotoxin S6c. The shaded circles indicate the amino acids that differ from that of endothelin-1. The lines indicate disulphide bridges linking cysteine amino acids between residues 1 & 15 and 3 & 11.



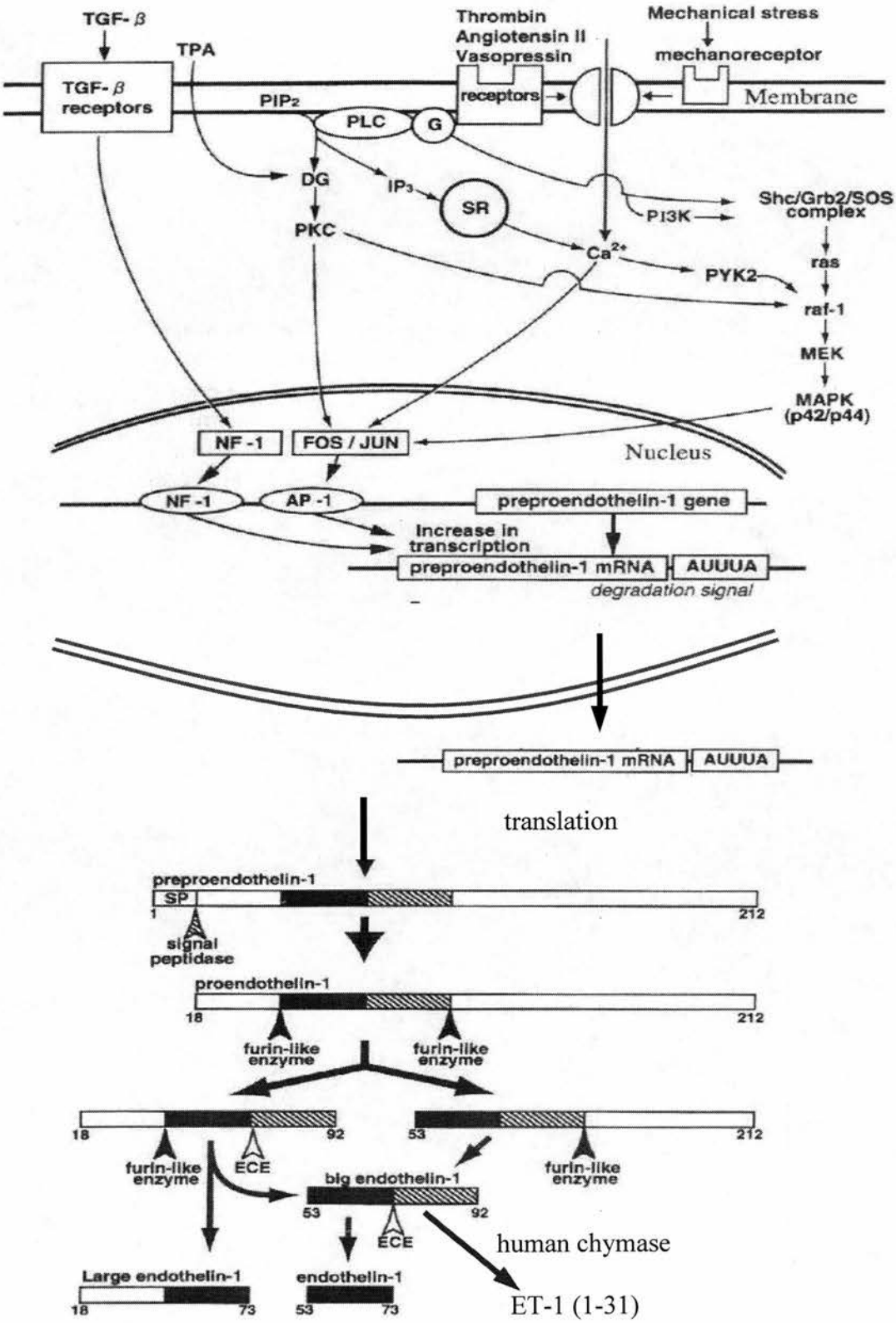
It is now also clear that the ET isopeptides share a close structural similarity to the sarafotoxins (SRTXs; see *Figure 1.3*), peptides isolated from the venom of the Israeli burrowing asp *Atractaspis engaddensis* (Kloog *et al.*, 1988). The ETs and the SRTXs act through common receptors to evoke a multitude of effects. Isoforms of SRTX have also been utilised extensively as tools for the characterisation of ET receptors (Sokolovsky, 1994). Though there are four identified isopeptides in the ET family, studies in this thesis will investigate the actions of the ET-1 peptide only.

1.2.2 Regulation of ET-1 synthesis

Extracellular factors can influence ET-1 generation both positively and negatively through a series of intracellular mediators that modulate gene transcription. PreproET-1 mRNA expression can be activated in endothelial cells and cardiomyocytes in response to such stimuli as hypoxia (Hu *et al.*, 1998), growth factors (Battistini *et al.*, 1993), cytokines (Molet *et al.*, 2000), thrombin (Golden *et al.*, 1998), Ang II, vasopressin and noradrenaline (Imai, T. *et al.*, 1992; Hanehira, T. *et al.*, 1997; McEwan, P.E. *et al.*, 2000).

Several recent studies also suggest that activation of ET-1 is associated with a deficiency of the NO pathway. Kourembanas *et al.* (1997) demonstrated that suppression of baseline NO levels *in vitro* using a NOS inhibitor in endothelial cells from normotensive rats led to a 3-fold increase in baseline ET-1 levels. Increased ET-1 gene expression was further demonstrated via blocking of endogenous NO using a NOS inhibitor in hypertensive rats *in vivo* (Tharaux *et al.*, 1999). Interestingly, shear stress has been shown to induce a sustained suppression, preceded by an early transient upregulation, in ET-1 gene expression in endothelial cells (Malek *et al.*, 1999). Stimulation of cGMP-mediated inhibition of phosphatidylinositol metabolism has been implicated in inhibition of ET-1 synthesis by heparin (Yokokawa *et al.*, 1993), atrial and brain natriuretic peptides (Hanehira *et al.*, 1997) and by the prostanoids prostaglandin E_2 and prostacyclin (Prins *et al.*, 1994). There are several regulatory pathways through which external factors can activate intracellular ET-1 gene expression *(Figure 1.4)*. The human ET-1 gene

Figure 1.4 Schematic pathway illustrating ET-1 synthesis. Modified from Miyauchi & Masaki: Annual Reviews in Physiology, 1999, 391-415.



contains five exons that encode preproET-1. Several regulatory elements are found in the 5' region of the ET-1 gene: motifs of the consensus binding sequence for the transcription factor nuclear factor (NF)-1 (Gronostajski, 1987), and sequences highly homologous to the octanucleotide consensus AP-1/Jun-binding site (Inoue *et al.*, 1989). The presence of an AP-1/Jun-binding site in the 5'-flanking region could explain the rapid induction of ET-1 as demonstrated following treatment of endothelial cells with the protein kinase (PK) C activator phorbol ester (reviewed by Miyauchi & Masaki, 1999).

ET-1 mRNA released from the nucleus into the cytoplasm, translates a 212 amino acid polypeptide termed preproET-1. PreproET-1 is cleaved by a signal peptidase to produce proET-1 which is further cleaved by a furin-like enzyme at two recognition sequences (Lys52-Arg53 and Arg90-Arg91), resulting in the 38 amino acid peptide big ET-1. The mature ET-1 is then generated through an unusual proteolytic cleavage between Trp21-Val22 of big ET-1 by another endopeptidase endothelin-converting enzyme (ECE; ET-1 synthesis pathway reviewed by Miyauchi & Masaki, (1999)). This hypothesis was supported by the presence of big ET-1 and its carboxyl terminal fragment in the conditioned medium of endothelial cells, indicating that all of the biosynthetic stages can occur within the endothelial cell (Emori *et al.*, 1989).

Big ET-1 is several orders of magnitude less active than ET-1 for displacement of binding to ET receptors and also in stimulating vascular constriction *in vitro* (Hirata *et al.*, 1990; Lal *et al.*, 1998). The conversion of big ET-1 to ET-1 is essential for biological activity, because the pressor action of big ET-1 is almost completely inhibited by a relatively large dose of phosphoramidon, an inhibitor of ECE (Matsumura *et al.*, 1990, Haynes & Webb, 1994). Because of the difference in activity between ET-1 and big ET-1, inhibition of ECE has been proposed as a possible therapeutic intervention on the actions of ET-1.

ECE was first purified to homogeneity from rat lung (Takahashi *et al.*, 1993). Cloning of the rat, human and bovine cDNAs to ECE-1 rapidly followed (Ikura, T. *et al.*, 1994; Schmidt, M. *et al.*, 1994; Shimada, K. *et al.*, 1994) with ECE-1 being identified as a zinc metallopeptidase related to neutral endopeptidase (Turner & Murphy, 1995). ECE-1 is a type-II integral membrane protein composed of 754 or of 758 amino acids, with a large C-terminal domain containing the enzymatic active site and a short N-terminal cytoplasmic tail, and has been shown to be localised on the cell surface as well as intracellularly (Barnes *et al.*, 1996; Russell *et al.*, 1998). Variability of the ECE-1 short N-terminal domain is responsible for the existence of different isoforms (ECE-1a, ECE-1b and ECE-1c) which cleave big ETs with identical efficiency but differ with respect to their subcellular localisation (Schweizer *et al.*, 1997). The existence of these isoforms in the human is the direct consequence of the presence of independent promoters on the ECE-1 gene (Valdenaire *et al.*, 1995; Orzechowski *et al.*, 1997). Furthermore, a recent study has reported the existence of a fourth novel human ECE-1 isoform, ECE-1d, with its own independent promoter region on the ECE gene and which is expressed on the cell surface (Valdenaire *et al.*, 1999). ECE-1a is in addition strongly expressed at the plasma membrane, which is not the case for ECE-1b, which is almost exclusively intracellular. ECE-1c is moderately expressed at the cell surface.

A second ECE subtype termed ECE-2 has also been cloned (Emoto & Yanagisawa, 1995). This is also a Type II integral membrane protein composed of 787 amino acids, but unlike ECE-1 has an acidic pH optimum. ECE-1 is widely distributed, but not found in neurons and glia in the brain, which are known to produce mature ETs (Xu *et al.*, 1994). ECE-2, in contrast, seems to be most abundantly expressed in neural tissues (Emoto & Yanagisawa, 1995). Two isoforms of ECE-2 have recently been isolated and may have different intracellular targeting and subcellular localisations (Nakahara *et al.*, 1999). The recently reported targeted disruption of the ECE-1 gene in mice, however, indicates that ECE-1 is the main enzyme responsible for the transformation of big ETs to ETs (Yanagisawa *et al.*, 1998).

Early evidence suggested that the conversion of big ET-1 to ET-1 *in vivo* occurred via ECE present on the plasma membrane. For example, Fukuroda *et al.* (1990) demonstrated that the conversion of big ET-1 to ET-1 in isolated blood vessels was dependent on an intact endothelium. The current consensus though, is that endogenous big ET-1 is most likely to be converted during its transit through intracellular constitutive and regulated secretory pathways, especially within the Golgi apparatus (Corder & Barker, 1999). This conclusion is consistent with immunohistochemical staining for ET-1 and big ET-1 in the cytoplasm of endothelial cells and the reported ability of a low density intracellular fraction to convert big ET-1 to the mature peptide (Gui *et al.*, 1993; Harrison *et al.*, 1995). Additionally, ET-1, big ET-1 and ECE have been located in vesicles within endothelial cells suggesting that these subcellular compartments are an important site for processing of big ET-1 by ECE (Harrison *et al.*, 1995; Ozaka *et al.*, 1997; Russell *et al.*, 1998). There are therefore two suggested secretory pathways in the secretion of ET-1: a constitutive pathway controlled at the level of protein synthesis and involves basal release of ET-1, and a regulated pathway involving rapid, stimulated degranulation of ET containing storage granules at the cell surface.

1.2.3 Endothelin receptors

The diverse biological functions exerted by ET-1 predicted the existence of more than one ET receptor. Two cDNAs that encode ET receptors were cloned from rat (Arai *et al.*, 1990) and bovine (Sakurai *et al.*, 1990) lungs, with further ET receptor cDNA cloned from rats (Lin *et al.*, 1991), pigs (Elshourbagy *et al.*, 1992), and humans (Sakamoto *et al.*, 1991; Hosoda *et al.*, 1992). These receptors could be classified into two groups, according to the relative binding affinities of the ET and SRTX isopeptides for the receptors. The order of affinity for the first receptor type, designated ET_A, was ET-1>ET-2>>ET-3>SRTX S6b. The second receptor type, designated ET_B, showed equal affinity for ET-1, ET-2 and ET-3 (Sakurai *et al.*, 1991).

Screening of amphibian cDNA libraries has revealed the existence of two other receptor clones, though neither has been detected yet in the mammalian genome. Emori *et al.* (1990) initially demonstrated an alternative ET-3 preferring receptor subtype. This receptor was subsequently cloned from *Xenopus* dermal melanophores and was termed the ET_C receptor (Karne *et al.*, 1993). The second, cloned from *Xenopus* heart, was termed ET_{AX} because of its high affinity for ET-1, like the ET_A receptor (Kumar *et al.*, 1994).

The ET_A and ET_B receptor genes located on chromosome 4 and 13 respectively, have similar structural organisation, suggesting that they originated from the same ancestral gene. Amongst the ET receptors, the 7 transmembrane domains and cytoplasmic loops of the receptors are highly conserved, but the N-terminal and other extracellular domains exhibit differences in both length and amino acid sequences. ET receptors can be divided into two distinct parts, one involved in ligand receptor binding comprising of transmembrane domains I, II, III and VII; the other comprising transmembrane domains IV, V and VI that determine isopeptide selectivity (reviewed by Gray & Webb, 1996).

In situ hybridisation and northern blotting techniques have revealed quite different distributions of the two receptors. The ET_A receptor is predominantly expressed in vascular smooth muscle, including aorta, coronary vessels and renal arterioles, but also in bronchial smooth muscle, myocardium and the adrenal and pituitary gland (Arai *et al.*, 1990; Hori *et al.*, 1992; Shibata *et al.*, 1997; Maxwell *et al.*, 1998). The ET_B receptor is most abundant in vascular endothelial cells and this might account in part for the substantial levels of ET_B receptor mRNA found in the brain, lung, kidney, stomach, liver, intestine and adrenal glands (Hori *et al.*, 1992; Elshourbagy *et al.*, 1993; Mathison & Israel, 1998).

ET receptors have been detected within the heart in various cells. Both ET_A and ET_B receptors were located on human and rat cardiomyocytes (Molenaar *et al.*, 1993; Kelso *et al.*, 1996), fibroblasts (Katwa *et al.*, 1993) and within the coronary vasculature (Opgaard *et al.*, 1996). There is also evidence of different ratios of ET receptors for specific cell types in the heart. Adult rat cardiomyocytes were found to express predominantly ET_A receptors (>90%); in contrast, both ET_A and ET_B receptor subtypes were nearly equally represented on cardiac fibroblasts (Fareh *et al.*, 1996). However, the overall ratio of ET_A to ET_B receptors in the normal rat heart has been reported to be around 90:10 (Sargent *et al.*, 1994; Sakai *et al.*, 1996a), though this ratio may change with the progression of some diseases.

ET receptor expression can be influenced by various factors including Ang II, cytokines, insulin and steroids (Nambi *et al.*, 1992; Kanno *et al.*, 1993; McDonald *et al.*, 1995; Uddman *et al.*, 1999). Changes in ET receptor expression have been demonstrated in various pathological conditions including ET receptor upregulation in the rabbit diabetic kidney (Khan *et al.*, 1999), the porcine coronary artery after restenosis (Katwa *et al.*, 1999), and in the lung and heart of the rat model of chronic hypoxic pulmonary hypertension (Li *et al.*, 1994). ET_B receptor downregulation has been reported in the aorta of DOCA-salt hypertensive rats (Bokil *et al.*, 1999). Additionally, prolonged exposure of cultured smooth muscle cells (Hirata *et al.*, 1988) and Swiss 3T3 cells (Devesly *et al.*, 1990) to ET-1 *in vitro* caused a marked

decrease in ET-1 binding sites, indicating that ET receptors can be down-regulated by ET-1 itself.

The development of potent and selective ET receptor agonists and antagonists (*Table 1.1*) has allowed the functional role of ET-1 to be more clearly defined (Allcock *et al.*, 1993; Bonvallet *et al.*, 1993; Karaki *et al.*, 1993).

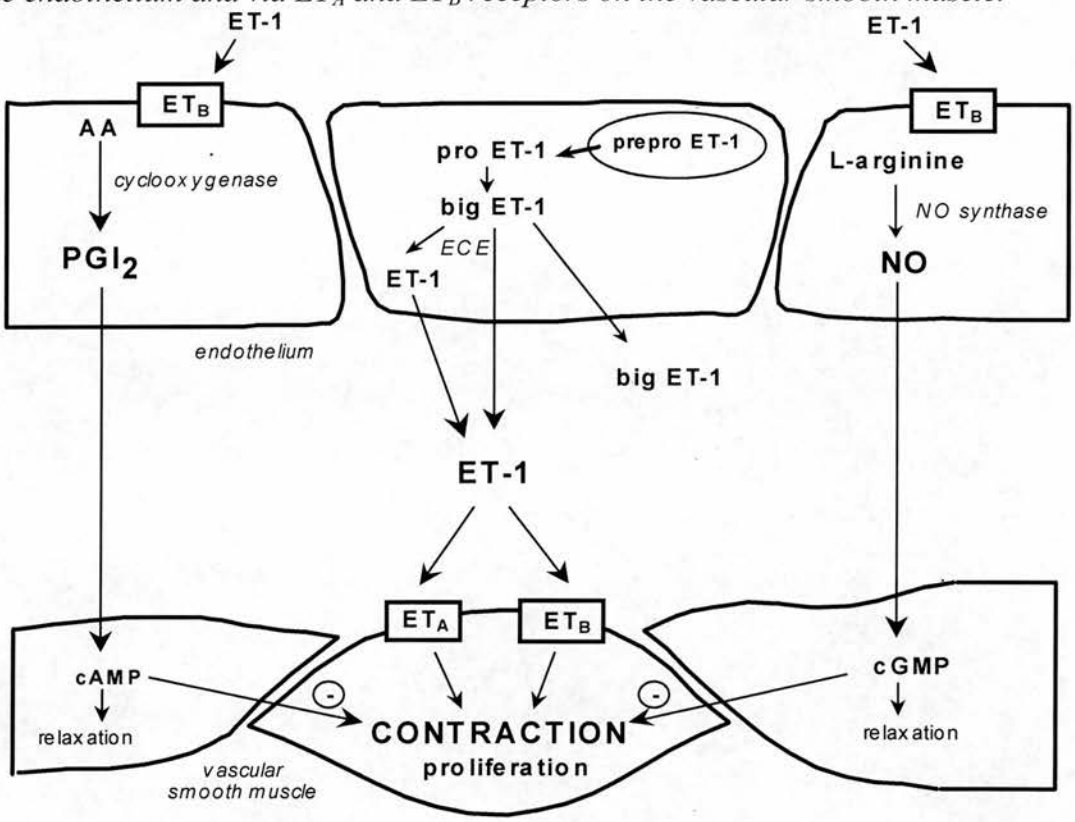
Table 1.1 List of some of the main ET receptor agonists and antagonists in experimental use.

Receptor	ET_A	ET_B
ET potency order	ET-1>ET-2>ET-3	ET-1=ET-2=ET-3
Selective agonists		Sarafotoxin S6c IRL1620 BQ3020
Selective antagonists	BQ-123 FR139317 LU135252 PD156707	BQ788 IRL2500 Ro468443
Non-selective	TAK-044 (peptide) Bosentan (nonpeptide) SB209670 (nonpeptide)	

The findings of the above agonist and antagonist studies are consistent with the view that constriction of vascular smooth muscle is mediated predominantly by ET_A receptors (*Figure 1.5*) and that constriction is modified by release of relaxing factors from the endothelium through stimulation of ET_B receptors (*Figure 1.5*). However, successive studies showed that *in vivo* pressor responses produced by ET-1 could not be inhibited completely by ET_A receptor antagonists (Cristol *et al.*, 1993; McMurdo *et al.*, 1993). ET_B receptor mRNA has been detected in both the medial smooth muscle of human arteries (Davenport *et al.*, 1993; Maguire *et al.*, 1994) and in cultured smooth muscle cells (Batra *et al.*, 1993). Furthermore, ET_B receptor agonists have been shown to evoke constriction *in vitro* (Sumner *et al.*, 1992; Shetty *et al.*, 1993; Gray *et al.*, 1995) and pressor responses *in vivo* (Clozel *et al.*, 1992; Haynes *et*

al., 1995a). These studies indicated that ET_B receptors are present on vascular smooth muscle cells where they mediate constriction (*Figure 1.5*). In addition, Mickley *et al.* (1997) demonstrated the relative role of ET_B-mediated vasoconstriction in rat small mesenteric arteries is greater when the ET_A-mediated effect is blocked, suggesting the presence of a possible 'crosstalk' mechanism existing between the receptors.

Figure 1.5 Diagrammatic representation of the actions of ET-1 via ET_B receptors on the endothelium and via ET_A and ET_B receptors on the vascular smooth muscle.



Additionally, use of ET receptor antagonists have suggested the presence of subtypes of the ET_A and ET_B receptor populations (Sudjarwo *et al.*, 1994; Douglas *et al.*, 1994). These authors proposed a subclassification based on the sensitivity of vasoconstrictor and vasodilator responses to BQ-123 and PD 142893. ET_A receptors could further be subclassified into ET_{A1} and ET_{A2} receptors, based on the susceptibility to an ET_A receptor antagonist BQ-123, in which ET_{A1} is sensitive to and ET_{A2} is resistant to BQ-123 (Sudjarwo *et al.*, 1994). The ET_B receptor was also subclassified as an ET_{B1} receptor, which mediates mainly vasorelaxation through the

release of nitric oxide and is inhibited by PD 142893 and bosentan, and the ET_{B2} receptor, which mediates direct vasoconstriction of veins and is inhibited by SB 209670 and BQ-788 (Douglas *et al.*, 1994).

Generally, activation of both ET_A receptors and ET_B receptors on vascular smooth muscle cells (Force, 1998) and cardiomyocytes (Sugden & Bogoyevitch, 1996) by ET-1 leads to a biphasic increase in intracellular calcium (Ca^{2+}). In the first phase, ET-1 binds to the $ET_{A/B}$ receptors at the cell surface activating a pertussis toxin-insensitive G-protein, which in turn stimulates phospholipase C (PLC). Hydrolysis of phosphatidylinositol by PLC generates inositol triphosphate (IP_3) and diacylglycerol (DAG), both of which function as second messengers transducing information from the surface of the cell to the interior. IP_3 is a fast-acting mediator that binds to a receptor on the sarcoplasmic reticulum and triggers the release of Ca^{2+} from intracellular stores. After the release of Ca^{2+}, a second phase is initiated in which Ca^{2+} moves into the cell from the extracellular space (Berridge, 1993), and it is this sustained elevation in intracellular Ca^{2+} that is responsible for increases in cell contractility. DAG activates PKC that sensitises the contractile proteins to Ca^{2+} via phosphorylation (Sunako *et al.*, 1989; Takanashi & Endoh, 1991). The increase in intracellular pH enhances the contractility of the cell, again via sensitisation of the contractile proteins via Ca^{2+}.

A recent interesting study has also indicated a role for ET-1 in stimulating ANP release in rat cardiomyocytes *in vitro* through ET_A receptor-mediated activation of cAMP. Rebsamen *et al.* (1997) further demonstrated that in these cells calcium mobilisation from intracellular stores and stimulation of cAMP production represented two cellular events linked to two different G proteins that are coupled to one receptor, the ET_A receptor.

ET_B receptors on endothelial cells in the heart stimulate a similar PLC-mediated intracellular pathway to that of ET_A and ET_B receptors on cardiomyocytes, but in endothelial cells the outcome is the release of a physiological endothelial-derived relaxing factor NO. Stimulation of ET_B receptors at the cell surface by ET-1 activates

a pertussis toxin-insensitive G-protein, which through a series of intracellular processes (as described above) leads to an influx of Ca^{2+} and an increase in intracellular Ca^{2+} levels. The enzyme NOS, which is central to NO biosynthesis is controlled by intracellular Ca^{2+}/calmodulin. A Ca^{2+}/calmodulin complex forms as a result of Ca^{2+} influx into the cell, which then binds to NOS; the resulting activated NOS leading to production and release of NO from endothelial cells.

Upon production, NO diffuses freely through the endothelial cell to cardiomyocytes where, depending on its concentration, it has biphasic contractile effects on cardiac tissue. High levels of NO induce large increases in cGMP (Ignarro, 1989). Raised cGMP levels has actions on myosin light chain kinase, PKG, phosphodiesterases and ion channels resulting in reduced intracellular Ca^{2+} (Moncada & Higgs, 1991) and a negative inotropic effect mediated by a PKG-dependent reduction in myofilament responsiveness to Ca^{2+}. Conversely, low levels of NO increase cAMP in part by a novel cGMP-independent activation of adenylyl cyclase and induces a positive contractile response (Kojda *et al.*, 1996; VilaPetroff *et al.*, 1999).

1.2.4 Clearance and Degradation of Endothelin

The observation that concentrations of circulating ET-1 were increased by a mixed ET_A/ET_B receptor antagonist (Loffler *et al.*, 1991) or by a selective ET_B receptor antagonist (Fukuroda *et al.*, 1994), but not by ET_A receptor antagonists, was suggestive of a role for ET_B receptors in the clearance of ET-1. ET_B receptors were also shown to be completely and exclusively responsible for pulmonary ET-1 removal in the dog *in vivo,* with a half-life of plasma ET-1 of 3-5 minutes (Dupuis *et al.*, 1996). As observed with other vasoconstrictor substances, internalisation of the endothelin receptor complex is believed to be the mechanism for down regulation and clearance (Resink *et al.,* 1990a). After internalisation, the ligand-receptor complex is thought to be sequestered into lysosomes, which the acidic environment promotes ligand dissociation (Hirata *et al.,* 1988). Bremnes *et al.* (2000) recently reported a similar possible mechanism for the clearance of plasma ET-1 by

demonstrating that ET_A and ET_B receptor subtypes were targeted to different intracellular routes after ET stimulation. Whereas the internalised ET_A receptor was recycled to the cell surface, the ET_B receptor followed an arrestin- and dynamin/clathrin pathway to intracellular lysosomes whereupon they were degraded.

1.2.5 Physiological actions of Endothelin in the cardiovascular system

1.2.5.1 Vascular Tone

In view of the multiple cardiovascular actions of ET-1 and the fact it is one of the most potent vasoconstrictor peptides known, there has been much interest in its contribution to basal vascular tone. The physiological importance of endogenous ET-1 in the maintenance of basal vascular tone and blood pressure in humans has been demonstrated by local (Haynes & Webb, 1994) and systemic (Haynes *et al.*, 1996) vasodilation in response to inhibitors of the ET system. More recently, Spratt *et al.* (1999) demonstrated that systemic blockade of the ET_A receptor, using BQ-123, inhibited agonist-induced vasoconstriction and decreased peripheral vascular resistance, suggesting that ET_A-mediated vascular tone contributes to the maintenance of basal systemic vascular resistance and blood pressure. Furthermore, in the forearm vasculature, there was a similar threshold for inhibition of vasoconstriction to both exogenously administered and endogenously generated ET-1. ET-1 mediated local and systemic vasoconstriction was also demonstrated using a specific ET_A receptor antagonist BMS 193884 given to human subjects, with orally administered antagonist effects still evident 24 hours later (Strachan *et al.*, 1999).

The sustained vasoconstrictor effects of ET-1 are predominantly mediated by the ET_A receptor, although vascular smooth muscle ET_B receptors may also contribute to ET-1 mediated vasoconstriction in animals (Clozel *et al.*, 1992) and humans (Haynes *et al.*, 1995b) *in vivo.* However, Strachan *et al.* (1999) recently demonstrated that systemic administration of the ET_B receptor selective antagonist, BQ-788, causes substantial systemic vasoconstriction associated with a reduction in heart rate and cardiac index, suggesting that the overall balance of effects of endogenous ET-1 at the vascular ET_B receptor favours vasodilatation. Through these ET receptor

antagonist studies, it is now acknowledged that ET-1 has a role to play in mediating vascular tone under physiological conditions through its receptors on both smooth muscle (ET_A and ET_B) and endothelial cells (ET_B).

1.2.5.2 Effects on the Heart

It is now established that the precursors of ET-1, ECE, ET-1 itself and its receptors are all present in the heart. Synthesis of ET-1 peptide has been reported in cultured neonatal rat cardiac myocytes (Suzuki *et al.*, 1993) and ischaemic porcine cardiac myocytes *in vivo* (Tonnessen *et al.*, 1995). ET-1 immunoreactivity and preproET-1 mRNA expression has also been identified in endothelial cells throughout human hearts (Plumpton *et al.*, 1996) and in endothelial cells and cardiomyocytes in human transplanted heart tissue (Giaid *et al.*, 1995). Furthermore, ECE immunoreactivity has been reported in endocardial endothelial cells of the heart (Davenport *et al.*, 1998).

Specific sarcolemmal receptors for ET-1 are present on cardiac myocytes isolated from mammalian (Hirata *et al.*, 1989; Ono *et al.*, 1995) and human hearts (Moody *et al.*, 1990). Both ET_A and ET_B receptors have been identified on cardiac myocytes (Molenaar *et al.*, 1993; Ono *et al.*, 1995) and are coupled to multiple subcellular signalling pathways including stimulation of phosphoinositide hydrolysis, activation of arachidonic acid metabolism, and G-protein mediated inhibition of adenylyl cyclase (Hilal-Dandan *et al.*, 1992; Clerk & Sugden, 1997).

ET_A and ET_B receptor antagonists have been shown to increase and decrease coronary flow respectively in isolated perfused rat hearts indicating the presence of both ET receptors on the coronary vasculature, and suggesting an indirect effect of ET-1 on cardiac muscle through modulation of coronary artery tone (Wang *et al.*, 1994). Systemically administered ET_A selective receptor antagonist BQ-123 in healthy human subjects was reported to increase heart rate and cardiac index (Spratt *et al.*, 1999) indicating a role for ET-1 through the ET_A receptor in control of basal cardiac parameters. Furthermore, infusion of the ET_A selective antagonist BQ-123 in healthy volunteers, caused a significant reduction in left ventricular dP/dt, with no

effect on relaxation suggesting that endogenous ET-1 may have a physiological role, contributing to cardiac output (MacCarthy *et al.*, 2000).

Shortly after ET-1 had been identified as an endothelium derived vasoconstrictor, Ishikawa and colleagues (Ishikawa *et al.*, 1988) also reported that ET-1 had a potent inotropic activity in guinea pig atrial strips. Subsequent studies have demonstrated positive inotropic effects of ET-1 in both perfused rat heart (Kusumoto *et al.*, 1996) and human atrial myocardium (Meyer *et al.*, 1996) *in vitro*. Interestingly, MacCarthy *et al.* (2000) demonstrated a small positive inotropic effect of exogenously administered ET-1 in normal human hearts *in vivo*, but a negative inotropic effect in heart failure, which may be due to differences in intracellular signalling or to excess ET-mediated vasoconstriction in the heart failure group. However, the effect of endogenous ET-1 on intrinsic contractile function of the failing human heart remains unknown.

In separate studies, both anti- and pro-arrhythmic effects have been suggested for ET-1. In isolated ventricular myocytes, ET-1 acting through ET_A receptors inhibited PKA and protected the ventricle against arrhythmias (James *et al.*, 1994). However, ET-1 has been reported to induce arrhythmic contractions in human isolated atrial tissue, which did not appear to be mediated by the ET_A receptor (Burrell *et al.*, 2000). Also, infusion of ET-1 through coronary vessels in isolated perfused rat and guinea pig hearts led to an increase in coronary perfusion pressure, where at peak pressure, ventricular arrhythmias were observed (Ercan *et al.*, 1996). Paradoxically, low dose of the ET_A receptor antagonist BQ-123 have been found to reduce the incidence of arrhythmias in the rat model of ischaemia, but a high dose of BQ-123 is pro-arrhythmic (Garjani *et al.*, 1995). However, at these higher doses, BQ-123 may no longer be ET_A specific. Furthermore, Sharif *et al.* (1998) demonstrated that ET-1 released endogenously during ischaemia is arrhythmogenic, whereas exogenous application of ET-1 under certain conditions is anti-arrhythmogenic.

Growth effects of ET-1 on the heart are discussed in section 1.2.6

1.2.5.3 Actions on the Kidney

The ETs are produced by, bind to, and induce biological responses in a wide variety of renal cell types (Kohan, 1997). While ET-1 is primarily implicated in regulating cell contraction or proliferation (Wang *et al.*, 1994; Nitta *et al.*, 1995) in the vasculature and mesangium, it serves a different function in the tubule. ET-1 may function as an autocrine inhibitor of water reabsorption in the collecting ducts (Tomita *et al.*, 1990; 1993). The peptide has a more complex effect on sodium reabsorption in the nephron; however, it is likely that the predominant effect on the proximal tubule, cortical collecting tubule and inner medullary collecting duct is to promote sodium excretion (Zeidel *et al.*, 1989; Tomita *et al.*, 1993; Ling, 1994). It is therefore apparent that ET-1 subserves opposite functions in the renal tubule and vasculature. In the vasculature, the peptide primarily reduces renal blood flow and glomerular filtration rate, thereby promoting sodium and water retention. Conversely, ET-1 appears to function as a diuretic and natriuretic agent in the nephron. Though there is abundant data indicating the potential of ET-1 in modifying renal hemodynamics and urinary salt and water excretion, it is still unclear if the peptide influences renal function under normal conditions.

Numerous studies indicate that ET-1 is involved in the pathogenesis of a broad spectrum of renal diseases including ischaemic renal failure, inflammatory glomerular nephritis and endotoxemia. ET-1 may also mediate, in part, excessive extracellular matrix accumulation and fibrosis occurring in chronic renal failure and diabetes mellitus (reviewed by Kohan *et al.,* 1997). Several clinical studies have indicated that in patients with systemic hypertension, progressive proteinureic nephropathies, or acute and chronic renal dysfunction due to cyclosporin A therapy, the endothelin pathway is activated, as previously observed in experimental animals. Continuing pre-clinical observations suggest strongly that ET antagonists are likely to have a promising role in the future treatment of renal dysfunction (reviewed by Benigni, 2000).

1.2.5.4 Endocrine Effects

ETs have been shown to play a pivotal role in the control of various endocrine and neuroendocrine tissues. The presence of ETs has been demonstrated in the porcine (Nunez *et al.*, 1990), rat (Sakurai *et al.*, 1991) and human (Imai *et al.*, 1992) adrenal gland. *In vitro* studies performed in several animal models have shown that ETs are potent stimulators of corticosteroid secretion. In rat, ET-1 caused a dose-dependent increase in both corticosterone and aldosterone secretion *in vitro* (Hinson *et al.*, 1991; Belloni *et al.*, 1995). Furthermore, ET-1 has been shown to regulate secretion of arginine vasopressin (AVP) and atrial natriuretic peptide (ANP) from the adrenal medulla. Macchi *et al.* (1999) demonstrated ET-1 to stimulate release of AVP and ANP from rat adrenal medulla fragments *in vitro,* with ET-1 acting exclusively via the ET_A receptor subtype.

ET-1 has also been found to exert multifaceted effects on the renin-angiotensin-aldosterone system (RAAS), such as dose dependently inhibiting renal renin production, directly stimulating aldosterone production from the adrenocortical zona glomerulosa and promoting growth of the adrenal cortex (reviewed by Rossi *et al.*, 1999). The observation that, despite this inhibitory effect on renin, ET-1 was found to markedly stimulate aldosterone secretion both in animals (via ET_B receptors) and in humans (via ET_A and ET_B receptors) suggests a direct effect of the peptide on the adrenal cortex.

It has been hypothesised that Ang II, the active peptide of the RAAS, affects the synthesis of ET-1, which in turn can influence the RAAS by acting at different steps. Herizi *et al.* (1998) demonstrated that bosentan entirely prevented the exogenous effects of a ten day infusion of Ang II (200ng/kg/min) including development of hypertension, the reduction of renal blood flow and a marked increase in heart weight. Furthermore, bosentan was found to exert a hypotensive effect in addition to that of the AT_1 receptor antagonist losartan in a canine model of hypertension (Massart *et al.*, 1998).

An animal model that lends itself to the investigation of the interactions between the RAAS and ET systems *in vivo* is the TGR(mREN-2)27 rat. In this model, the introduction of the Ren-2 gene, encoding for renin, causes severe hypertension, associated with overexpression of the transgene in many tissues with ensuing enhanced endogenous production of Ang II in tissues with low plasma renin activity (Ganten *et al.,* 1991). It has been reported that aortic responsiveness to ET-1 followed changes that closely paralleled those of systolic blood pressure in these animals, thereby suggesting a role for ET-1 in this form of renin-dependent hypertension (Cargnelli *et al.,* 1998). However, administration of the non-selective ET receptor antagonist bosentan did not have either a blood pressure lowering effect or any effectiveness in preventing left ventricular and vascular hypertrophy in male TGR(mREN-2)27 rats. The reasons why ET-1 may take part in hypertension induced by exogenously administered Ang II, but not in the models with an enhanced endogenous production of Ang II, are unclear at present.

In summary, there are several interactions between the RAAS and the ET system, which may have relevant effects on blood pressure and on hypertension-related complications. However, the question of whether or not they are also relevant in humans in the clinical setting and have therapeutic implications needs further investigation.

1.2.5.5 Interactions with the Nervous System

Specific distribution of ET within the central nervous system (CNS) has been demonstrated in many areas of the brain as well as the spinal cord (Kuwaki *et al.*, 1994). This extensive distribution suggests different roles for the ET system in CNS regulation of multiple physiological events including a role for the ET system in CNS regulation of cardiovascular function (Kuwaki *et al.*, 1995). With regard to the peripheral nervous system, ET receptors are found distributed in superior cervical and nodose ganglia as well as the carotid body, implicating an additional cardiovascular regulatory role by influencing both systemic arterial baroreceptor and chemoreceptor reflexes (reviewed by Mortensen, 1999).

1.2.6 Growth and mitogenic properties of ET-1

ET-1 has emerged as an important growth factor and as a substance with an obligatory role in normal tissue development and differentiation. Evidence indicating this comes from studies using mice in which the ability to synthesise ET-1, ET_A or ET_B receptors has been knocked out. In ET-1 gene knockout mice (Kurihara *et al.*, 1994), the homozygotes die at birth with abnormalities in craniofacial tissues and other organs that make breathing impossible. These malformations are also seen in ET_A receptor knockout mice (Yanagisawa *et al.*, 1998), indicating that endogenous ET synthesis leading to ET_A receptor activation is essential for normal development and survival. In contrast, gene targeting of ET_B receptor synthesis gives rise to mice that suffer from aganglionic megacolon, as seen in Hirschsprung's disease (Hosoda *et al.*, 1994). It is therefore evident that ET-1 and ET receptor genes are critical to normal development.

ET-1 is also an important regulator of mitogenesis influencing DNA synthesis, the expression of protooncogenes, cell proliferation and hypertrophy. ET-1 has been shown to regulate DNA synthesis in various cell types such as bovine brain capillary endothelial cells (Vigne *et al.*, 1990), rat kidney fibroblasts (Yeh *et al.*, 1991), cultured neonatal rat cardiomyocytes (Ito *et al.*, 1991) and rat vascular smooth muscle cells (VSMC; Rosen *et al.*, 1999). ET-1 also promoted VSMC proliferation (Peiro *et al.*, 1995) and induced hypertrophy of adult rat cardiac myocytes *in vitro* (Mullan *et al.*, 1997).

In vertebrates, the actions of many stimuli resulting in proliferative or hypertrophic growth converge on a set of cellular kinase cascades that are collectively called the mitogen-activated protein (MAP) kinase cascades (Force & Bonventre, 1998). Of the three MAP kinase cascades, the extracellular regulated kinase (ERK) cascade is critical to the mitogenic response, to cellular differentiation and, in some cells, to induction of hypertrophy. The other two MAP kinase cascades (the stress activated protein kinase / janus kinase cascade and the p38 cascade) are activated by cellular stress and cytokines. Growth factor stimulation (i.e. ET-1) of G-protein-coupled

surface receptors on cardiomyocytes leads to activation of internal signal transduction pathways and eventual activation of ERK. During prolonged activation, a portion of the ERK protein translocates to the nucleus (Gonzalez *et al.*, 1993; Lenormand *et al.*, 1993), and promotes cell hypertrophy by activating c-jun proto-oncogene expression (Davis, 1995). C-jun has been demonstrated to be an important growth factor in the myocardium (Schunkert *et al.*, 1991).

ET-1 has also been shown to co-operate with other growth factors in a synergistic fashion to enhance mitogenesis of fibroblasts and VSMCs *in vitro* (Weissberg *et al.*, 1990; Yeh *et al.*, 1991, Hafizi *et al.,* 1999). Bonin *et al.* (1993) reported that pre-treatment of human smooth muscle cells with either endothelial growth factor (EGF) or basic fibroblast growth factor (bFGF) produces significant increases in ET-1 binding to these cells without any significant change in k_d. A further interesting study by Hafizi *et al.* (1996) demonstrated pronounced synergy between ET-1 and three polypeptide growth factors, platelet derived growth factor (PDGF), bFGF and EGF, in co-stimulation of DNA synthesis in quiescent human cultured coronary artery smooth muscle cells. Since ETs themselves have been reported to elicit relatively weak mitotic responses (Battistini *et al.*, 1993), the synergistic effect between ET-1 and other growth factors may suggest a mechanism by which ET-1 acts as a regulator of growth and proliferation.

1.3 Heart failure

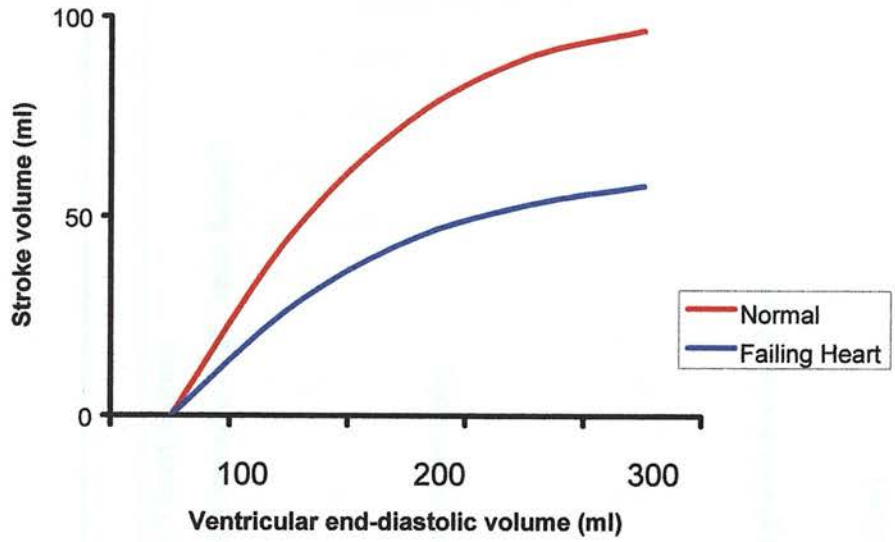
1.3.1 Aetiology of heart failure

Heart failure is somewhat difficult to define though it can be simply thought of as a clinical syndrome typified by an inability of the heart to meet the metabolic needs of the body and is recognised by a characteristic pattern of haemodynamic, renal, neural and hormonal responses.

Heart failure has, due to an increased ageing population, become more prevalent in recent years. There are almost 4.8 million people in the US with congestive heart failure (CHF), with this figure expected to reach 10 million by the year 2007 (Rich, 1997). More than $56 billion is spent annually on the treatment and management of heart failure, 70% of which covers the hospitalization of patients. However, mortality rates from CHF continue to be high, with a 6-year mortality rate secondary to heart failure of 84% in men and 77% in women (Croft *et al.*, 1999). There is therefore a need to develop innovative solutions and new treatments to keep this growing population out of hospital and improve overall survival.

In practice, heart failure may be diagnosed whenever a patient with significant heart disease develops the signs or symptoms of a low cardiac output, pulmonary congestion and systemic venous congestion. The cardiac output is a function of the preload (the volume and pressure of blood in the ventricle at the end of diastole), the afterload (the arterial resistance) and myocardial contractility. Starling's Law states that the more myocardial fibres are stretched (the end-diastolic fibre length), the greater the force of the ensuing contraction (see section 1.1.2.2). In heart failure, however, the curve moves to the right and becomes much flatter (*Figure 1.6*). Because of reduced contractility due to loss of contractile tissue, a given amount of work is only achieved with a greater end-diastolic fibre length. The reduced physiological limits of the myocardium and increased end-diastolic filling pressure leads to an overall lower cardiac output with increases in filling pressure.

Figure 1.6. The Frank-Starling curve for a normal heart (red), and during heart failure (blue).



There are many mechanisms for myocardial failure (*Table 1.2*) including pressure overload, volume overload, and reduced ventricular contractility. In each case, through different mechanisms, the myocardium attempts to compensate for the primary defect before the stages of overt myocardial failure develop.

In response to pressure overload, for example, via aortic stenosis or sustained severe hypertension, left ventricular (LV) pressure must increase to overcome the resistance to the ejection of blood. The mechanism for the increase at the cellular level is probably a stretch-induced increase in force of contraction (inotropy), controlled by mechanoreceptors. The result is that the LV systolic pressure increases, the obstruction to the outflow of blood is overcome, and cardiac output is maintained. The disadvantage of this mode of adaptation is that the LV wall stress is greatly increased, which may lead to LV dilation via increased transmural wall pressure, causing further increased wall stress. However, exposed to a sustained pressure load, the myocardium adapts via hypertrophy, and the wall becomes thicker. The result of hypertrophy is that the abnormally increased wall stress is reduced so that systolic

Table 1.2 Varying causes of heart failure with examples.

Type	*Examples*
Pressure Overload	Aortic stenosis, arterial hypertension (left heart failure)
(Ventricular outflow obstruction)	Pulmonary hypertension, pulmonary valve stenosis (right heart failure)
Restricted filling	Mitral / Tricuspid stenosis
(Ventricular inflow obstruction)	Constrictive pericarditis
	Endomyocardial fibrosis/ left ventricular hypertrophy
Ventricular volume overload	Aortic / mitral regurgitation
	Ventricular / atrial septal defect
	Some types of congenital heart disease
Reduced ventricular contractility	Hypertrophic / dilated cardiomyopathy (global dysfunction)
	Myocarditis (global dysfunction)
	Myocardial infarction (regional dysfunction)

function at rest is normal. However, such compensatory hypertrophy is gained at the cost of greater susceptibility to ischaemia and abnormal diastolic function.

The initial event in volume overload may be, for example, valvular regurgitation of either the mitral valve or the aortic valve. To deal with this volume load, there are both changes in the loading conditions and in ventricular size. First, the volume load means that the preload increases and the heart is functioning at the length limit of the Frank-Starling curve. Second, some increase in chamber volume may be attained by slippage of cells. The result is enhanced diastolic filling and decreased LV stiffness (Zile *et al.*, 1993), so that diastolic function improves rather than deteriorates as in pressure overload. Nonetheless, as chamber size increases, so must wall tension. The consequence is some hypertrophy, which allows the LV cavity to regain a normal wall stress by modest and proportional LV hypertrophy.

Where reduced ventricular contractility occurs, the dysfunction may be global (myocarditis or dilated cardiomyopathy) or regional (myocardial infarction, MI). In Western countries, coronary artery disease (CAD) is by far the main cause of heart failure due to left ventricular dysfunction, with dilated cardiomyopathy being much less common (McMurray *et al.*, 1992). CAD most often results from atherosclerosis, where fatty streak formation on the inside wall of the artery leads to formation of a plaque. The initial stage consists of an asymptomatic period during which non-obstructive plaque is formed with further progression depending on its associated risk factors. The second stage, however, consists of a rapid thrombogenesis due to plaque rupture exposing thrombogenic components such as collagen, platelets and tissue thromboplastin, which promote platelet aggregation, fibrin formation, and development of occlusive or near occlusive thrombus (Falk, E. *et al.*, 1996). CAD and its complications (including arrhythmia, angina pectoris and MI) are among the leading causes of death in the UK.

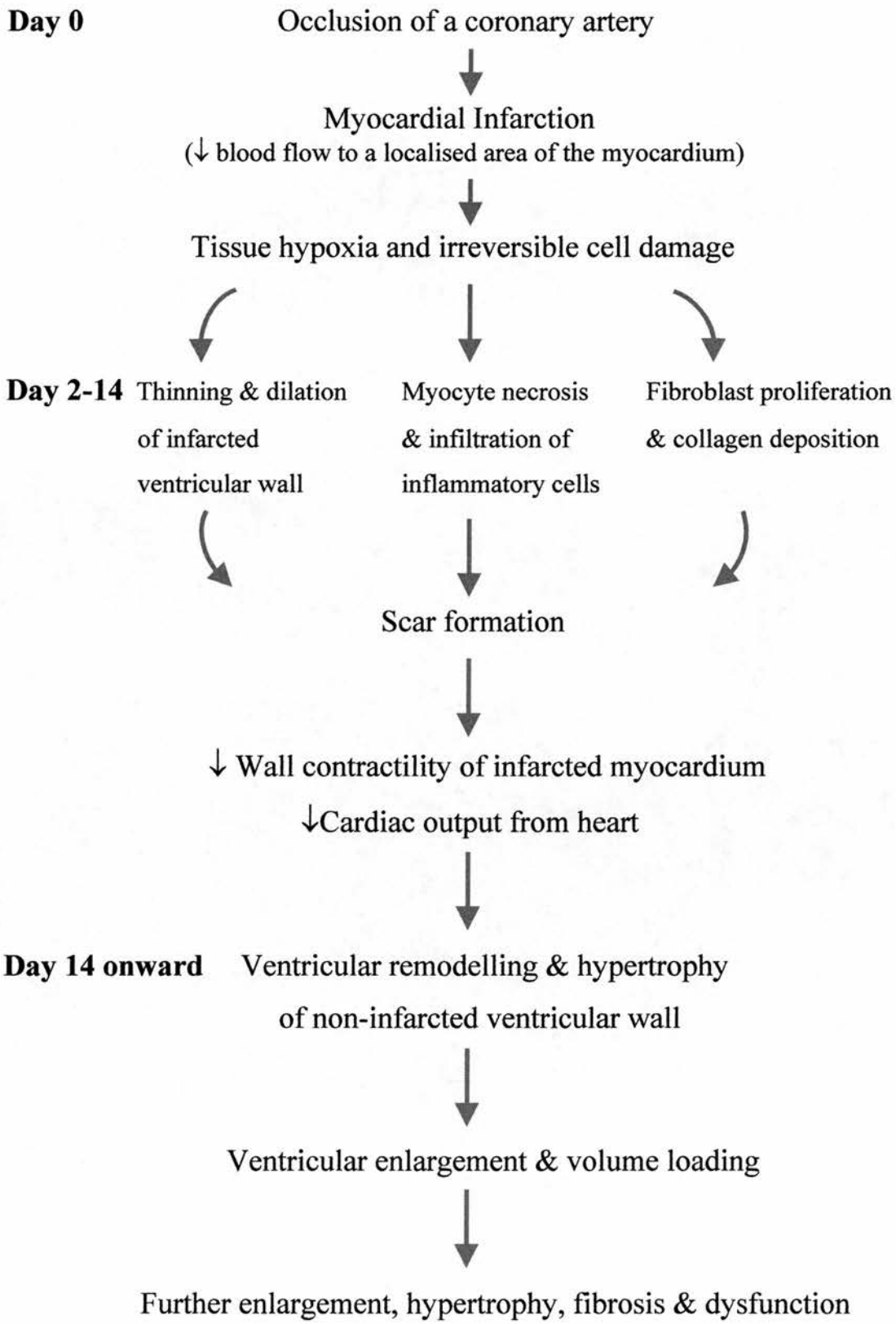
1.3.2 Acute phase after myocardial infarction

A left ventricular MI (for example secondary to coronary artery disease) starts a chain of events in the myocardium that aim to compensate for decreased contractility and cardiac output (*Figure 1.7*). The size of the MI is influenced by the localisation of the coronary artery occlusion, duration of ischaemia, left ventricular oxygen consumption during ischaemia and collateral blood flow (Maroko *et al.*, 1971; Schaper & Pasyk, 1976).

Initially, impaired coronary blood flow and oxygen supply to the affected area of myocardium produces extensive biochemical and morphological changes in the myocardial cells (Poole-Wilson, 1989). These changes, depending on the duration of ischaemia, result in widespread abnormalities of cardiac contraction, relaxation, electrical repolarisation and to varying degrees of myocardial damage. Acute deprivation of oxygen delivery to myocardial cells will, after 10-15 seconds, cause a reduction in oxygen tension and high-energy phosphates such as creatine phosphate and adenosine triphosphate. Myocardial metabolism changes from normally being aerobic to an anaerobic state. The activation of anaerobic glycolysis and the formation of lactate result in the reduction of intracellular pH (Peiper, *et al.*, 1980) and the subsequent accumulation of CO_2 and lactic acid in the interstitium. A prolonged period of ischaemia, myofibrillar oedema, reduced glycogen content and accumulation of lactic acid finally results in signs of irreversible myocardial cell damage and death.

There is evidence that apoptosis is a major source of myocyte cell death in the irreversibly injured myocardium (in both animals and humans), followed by necrosis at later time points (Kajstura *et al.*, 1996; Olivetti *et al.*, 1997; Buja & Entman, 1998). Apoptosis has been reported in the rat to begin within the first 2 hours after MI, where activation of an endogenous endonuclease results in endonucleolysis and the removal of apoptotic bodies by neighbouring cells without initiation of an inflammatory response (reviewed by Anversa *et al.*, 1998). Hallmarks of apoptosis include cell shrinkage, membrane blebbing, chromatin condensation, and DNA fragmentation (MacLellan & Schneider, 1997).

Figure 1.7 Flow diagram (including approximate time scale) of responses to a MI in the rat heart.



Where apoptosis is thought of as controlled physiological cell death, necrosis can be termed non-physiological cell death. Cell necrosis also commences 2-4 hours after MI and is characterised by an immediate loss of plasma membrane integrity, mitochondrial alterations, stimulation of an inflammatory cell infiltration (Darzynkiewicz *et al.*, 1992). Kajstura *et al.* (1996) reported a 2.4 fold increase in myocyte apoptosis in the infarcted portion of the rat wall 2 to 4.5 hours after coronary artery ligation, with levels decreasing after 1 day. Also, necrotic myocyte cell death increased continuously from 2 hours to 1 day but was markedly attenuated at 7 days.

Necrotic cells in the infarcted area release a number of substances (i.e. tumour necrosis factor (TNF)-α, interleukin-1, complement) that act as chemoattractants to inflammatory cells, thereby initiating an inflammatory response (Ono *et al.*, 1998). The traditional interpretation of inflammatory cells is that they function as a protective response to infection by ingesting and destroying infectious agents or scavenging dead tissue, thus paving the way for repair of the damaged site (Iverson, 1989). Neutrophils primarily enter the infected area where they interact directly with necrotic tissue and release toxic components such as neutrophil proteases and reactive oxygen species, which have cytotoxic effects on the surrounding tissue (Entman *et al.*, 1990). Soon after, macrophages and lymphocytes infiltrate the myocardium starting a process which catabolises and disposes of dead myocytes.

The process of degradation and clearance of necrotic tissue is followed by formation of a scar composed principally of collagen. Fibroblasts are attracted to the site of injury where they convert to myofibroblasts. Through a complex series of molecular events that includes expression of immediate early response genes (e.g. c-fos, c-jun and erg-1) and activation of multiple second messenger systems, which act synergistically to induce mitosis, these cells proliferate and lay down fibrillar collagen that replace lost myocytes (Weber, 1997). Myofibroblasts are central to fibrogenesis at sites of repair, synthesising type III and then type I collagen, the major fibrillar collagens that contribute fibrous tissue (Weber *et al.*, 1988; Bishop *et al.*, 1990). Northern blot and *in situ* hybridisation analyses have shown an increase in

type III procollagen mRNA at the infarct site by day 2 post–MI in the rat, reaching a peak at day 21 and declining thereafter (Cleutjens *et al.*, 1995). Type I procollagen increased at day 4 and remained elevated at week 4 at the site of the infarct (Cleutjens *et al.*, 1995). Myofibroblasts also contain actin filaments, which may enable these cells to reorganise the scar and hence be responsible for scar contraction (Whittaker, 1997).

Myofibroblasts express receptors for Ang II, transforming growth factor (TGF)-β_1, and endothelins, which permit their response to these regulatory molecules. TGF-β_1 has numerous actions on the extracellular matrix including chemotaxis, phenotype transformation, and proliferation of fibroblasts and scar tissue remodelling (Singer, *et al.*, 1986; Desmouliere, *et al.*, 1993). Macrophages, clustered at sites of tissue injury, are a likely source of TGF-β_1 (Riches, 1996) that may be important to the appearance of the myofibroblast phenotype (Desmouliere *et al.*, 1993). Depending on the species, the entire process of scar formation is completed within weeks to months (Fishbein, *et al.*, 1978).

1.3.3 Adaptive changes in the heart after myocardial infarction

An acute MI, particularly one that is large and transmural, can also produce long-term alterations in the topology of both the infarcted and non-infarcted regions of the ventricle. The structural remodelling of the left ventricle after MI is a time-dependent process involving both the region of necrosis and the residual normal myocardium (Pfeffer & Braunwald, 1990).

Early post-infarction, regions of hypoxic necrosis may become over-stretched during ventricular contraction due to increased systolic tension of the viable myocardium and with sufficient repetition, this process leads to permanent structural changes in the ventricle (Michel *et al.*, 1995). The non-contracting infarcted region undergoing stretch and stress relaxation shows an overall thinning and dilation, the thinning a consequence of slippage between muscle bundles resulting in a reduction in the number of myocytes across the infarcted region. The morphological consequences of this repetitive stretching of the myocardium during systole has being termed 'infarct expansion' and is associated with an elongation of the contractile zone with further distortion of the ventricular shape and increase in ventricular filling volume (Swan, 1993).

Later post-infarct, an overlapping phase of fibroblast proliferation and collagen deposition continues and completes the formation of scar tissue, wherein the remaining viable myocytes within the infarcted region are realigned and attached in a dense collagen matrix, providing resistance to further stretching (Vracko *et al.*, 1989). The fibroblast proliferation and collagen deposition is responsible for a decreased compliance in the infarcted area compared to the remaining functional myocardium in the non-infarcted areas (Pfeffer, 1995). To compensate for loss in wall contractility and overall dysfunction in the infarcted area of myocardium, the heart undergoes important morphological processes such as ventricular remodelling and hypertrophy in areas away from the infarct.

Post infarct remodelling is a regional process that progresses from adjacent non-infarcted regions to involve the entire left ventricle as further contractile units become exposed to the damaging effects of mechanical overload and/or humoral over stimulation (Sakai *et al.*, 1998). Analysis of cell signalling mechanisms shows that stretch activates multiple signal transduction pathways, similar to those activated by growth factors (Swynghedauw, 1999). The hypertrophic response is characterised by increased cardiomyocyte cell volume, proliferation of non-myocyte cells (mainly fibroblasts), activation of myosin light chain 2, skeletal α-actin and β-myosin heavy chain (Rumberger, 1994). The ventricular enlargement process leads to restoration of pump function without concomitant increases in filling pressure (McKay *et al.*, 1986).

The downside of the initial compensation which uses the altered geometry (enlargement) to restore stroke volume is that architectural changes create greater loading conditions on the viable myocardium that promote further enlargement, hypertrophy and dysfunction. Therefore, although appropriate myocardial hypertrophy of the surviving myocardium compensates for myocardial loss in the infarcted area, excessive hypertrophy of the myocardium is believed to be maladaptive and to be associated in part with dysfunction of the surviving myocardium (Sakai *et al.*, 1998). The transition from compensated to decompensated heart failure is also likely to be related to the overexpression of neurohormones and peptides such as noradrenaline, Ang II, ET-1, and pro-inflammatory cytokines (Piano *et al.*, 1998).

These pathological developments of decompensated heart failure translate into distinct, if overlapping, stages in clinical presentation and can be classified according to the New York Heart Association (NYHA) scaling system. This classification was first proposed in 1964, and updated in 1973. Patients are categorised as NYHA Class I, II, II or IV (*Table 1.3*). Classes II, III and IV are regarded as mild, moderate and severe heart failure respectively, patients in class I are effectively normal (Timms & Davies, 2000).

Table 1.3 NYHA grading of symptoms.

Class	Symptoms
I	Cardiac disease but without resulting limitation of physical activity.
II	Cardiac disease but with slight limitation of physical activity, comfortable at rest. Ordinary physical activity results in fatigue, palpitation, dyspnoea or anginal pain.
III	Cardiac disease resulting in marked limitation of physical activity but comfortable at rest. Less than ordinary physical activity causes fatigue, dyspnoea or anginal pain.
IV	Cardiac disease resulting in the inability to perform any physical activity without discomfort, often discomfort at rest. If any physical activity is undertaken, discomfort is increased.

1.3.4 Neurohormonal systems activated during heart failure

The body responds in a number of ways to the changes affected by a MI and are largely designed to compensate for reduced flow of blood to the body. Two sets of neurohormones with opposing effects are activated in CHF. The vasoconstrictor hormones including noradrenaline, Ang II, and AVP are anti-diuretic and, generally, have growth promoting properties. The vasodilator hormones (including the natriuretic peptides), on the other hand, are natriuretic, diuretic and have antimitogenic properties.

1.3.4.1 Increased adrenergic activity

In heart failure, decreased cardiac output leads to a number of adaptive mechanisms including activation of the sympathetic nervous system. Augmented sympathetic activity in heart failure is initially beneficial, increasing cardiac output (via activation of β_1 adrenoreceptors in the heart) and redistributing blood flow from the splanchnic area to the heart and skeletal muscles. In addition, renal vasoconstriction leads to salt and water retention, which may help to improve the perfusion of vital organs.

However, sustained sympathetic stimulation, as seen in heart failure, activates the RAAS and other neurohormones leading to progressive salt and water retention, vasoconstriction, oedema and increased pre- and afterload (reviewed by Ferrari *et al.*, 1998). The increase of adrenergic drive is characterised by decreased baroreceptor sensitivity, increased plasma noradrenaline levels, cardiac noradrenaline spillover and desensitisation of postsynaptic β-adrenoreceptor signal transduction. Furthermore, heightened activity of the adrenergic nervous system also leads to stimulation of myocardial α_1-adrenoreceptors, which may cause hypertrophy, re-expression of fetal genes (Bishorpic *et al.*, 1987), and the induction of peptide growth factors (Takahashi *et al.*, 1994).

1.3.4.2 The renin-angiotensin-aldosterone system

A decrease in blood pressure in normal subjects will release renin, an enzyme involved in the Ang II synthesis pathway. In CHF, both a low renal perfusion pressure and increased β-adrenergic stimulation contribute to increased renin release. The result is an Ang II mediated vasoconstriction to add to that mediated by the activated sympathetic nervous system. Ang II directly constricts the peripheral vessels and enhances the degree of sympathetic activation, while also evoking the release of aldosterone, leading to an overall increase in body fluid volume as well as retention of sodium and water. An increased circulating blood volume thus supports the maintenance of the cardiovascular homeostasis. However, continued activation of the RAAS leads to excessive elevation of systemic vascular resistance, increased volume retention precipitating oedema, increased preload and overall increased workload of the heart.

Previous studies propose the existence of a tissue-based RAAS, which suggests that Ang II can be produced locally and thus also act locally as a vasoactivator. All of the necessary components of the RAAS are present in several organs and tissues, including the vasculature, heart, and kidneys. In myocardium from animals with experimental heart failure, there is increased expression of ACE (Hirsch *et al.*, 1991; Schunkert *et al.*, 1990) and angiotensinogen (Lindpaintner *et al.*, 1993). Although

cardiac renin mRNA expression is extremely low, renin activity is readily detectable in the heart (Vonlutterotti *et al.*, 1994). In contrast to renin, angiotensinogen appears to be synthesised locally within the myocardium. Under normal physiological conditions, cardiac angiotensinogen levels are low. However, in rats with chronic pressure overload and in rats with left ventricular failure early after coronary artery ligation, cardiac angiotensinogen gene expression is upregulated (reviewed by Wollert & Drexler, 1999).

Angiotensin receptor (AT) expression has been shown to change in both animal studies and human heart failure. Nio *et al.* (1995) reported upregulated AT_1 and AT_2 receptor mRNA expression in the infarcted and noninfarcted portions of the left ventricle after coronary artery ligation in the rat. However, several groups investigating receptor expression in human heart failure have reported reduced AT_1 receptor density while AT_2 receptor density remained unaltered (Asano *et al.*, 1997; Haywood *et al.*, 1997).

Several observations suggest that a direct effect of Ang II on cardiac AT receptors may play a central role in modifying the structure and function of the myocardium in patients with heart failure by acting on a variety of cell types to promote cell growth and alter gene expression. In cardiac myocytes and fibroblasts obtained from the neonatal rat, Ang II caused myocyte hypertrophy and fibroblast proliferation associated with the expression of several early response genes (c-fos, c-jun, and c-myc), angiotensinogen and the peptide growth factor, TGF-β_1 (Sadoshima & Izumo, 1993; Crawford *et al.*, 1994). Ang II has also been reported to induce expression of fetal genes encoding atrial natriuretic factor and α-skeletal actin in myocytes (Sadoshima & Izumo, 1993). Furthermore, a study investigating the influence of chronic Ang II infusion *in vivo* on expression of preproET-1, ET_A and ET_B receptor mRNA in the rat heart, showed upregulation of ET-1 synthesis in the myocardium, as well as modification of ET receptor expression in the presence of selective AT_1 blockade, suggesting a role for the AT_2 receptor in regulating expression of the myocardial ET system (McEwan *et al.*, 2000).

1.3.4.3 Arginine vasopressin

AVP belongs to a family of vasoactive and mitogenic peptides and has multiple actions including inhibition of diuresis, contraction of vascular smooth muscle, trophic actions and platelet aggregation (reviewed by Burrell *et al.*, 2000). Recently, *de novo* synthesis of AVP has been identified in the rat heart (Hupf *et al.*, 1999), suggesting that the peripheral and central AVP systems may exist as separate independent systems.

The precise role of AVP in the pathophysiology of cardiovascular disease is controversial. However, circulating levels of AVP are elevated in heart failure (Naitoh *et al.*, 1998) and in low-renin hypertension (Bakris *et al.*, 1997). In CHF, AVP may contribute to water retention and hyponatraemia through effects at the renal V_2 receptor and to increased peripheral resistance through potent constrictor actions at the V_{1A} receptor. Therefore, the recent availability of orally active non-peptide vasopressin receptor antagonists may allow AVP receptor antagonism to be considered as a therapeutic option in cardiovascular disease.

1.3.4.4.Atrial and brain natriuretic peptides

The natriuretic peptides, including ANP and BNP, are upregulated in heart failure and counteract neurohormones that induce vasoconstriction and fluid retention. ANP induces both diuretic activities via inhibition of aldosterone secretion and vasodilation through inhibition of noradrenaline release from terminal neurons (reviewed by Stein *et al.*, 1996). In addition there is evidence that the natriuretic peptides may directly inhibit fibroblast and vascular smooth muscle hypertrophy and interstitial fibrosis (Itoh *et al.*, 1990; Cao & Gardner, 1995). However, increased levels of natriuretic peptides in CHF are overcome by the drives toward vasoconstriction and sodium retention resulting from RAAS activation. In addition, the atrial stretch receptors involved in the secretion of ANP become downgraded, and vascular ANP receptors decrease their sensitivity to circulating ANP stimulation (Ferrari & Agnoletti, 1989; Matsumoto *et al.*, 1999).

1.3.5 Current clinical treatments

1.3.5.1 Diuretics

Diuretics are generally prescribed for all patients with heart failure who have evidence of fluid retention, such as pulmonary congestion, raised jugular venous pressure, or peripheral oedema. The increased diuresis and consequent reduction of the expanded blood volume is accompanied by improvement in cardiac pumping performance due to the reduction in preload and afterload. However, in using diuretics, specific attention needs to be directed towards prevention of side effects that include electrolyte imbalances, activation of neurohormonal systems, hypotension, and interaction with other drugs (for example hyperkalaemia) when using potassium sparing diuretics in combination with ACE inhibitors.

1.3.5.2 ACE inhibitors and Ang II receptor antagonists

ACE inhibitors block the conversion of Ang I to Ang II and prevent the degradation of BK by ACE. They therefore alter the balance between the vasoconstrictive, salt retaining, and hypertrophic properties of Ang II and the vasodilatory and natriuretic properties of BK. In large randomised clinical trials, ACE inhibitors have been shown to improve ventricular function, prolong survival and reduce the need for hospital admissions in patients with heart failure (reviewed by Lonn & McKelvie, 2000). Adverse effects of ACE inhibitors such as hypotension, syncope, hyperkalaemia, and renal insufficiency can occur, with careful initiation of treatment and subsequent regular monitoring required. Furthermore, a non-productive cough may develop in some patients via an ACE inhibitor-mediated increase in bradykinin.

Ang II receptor antagonists (ARAs) can be used as an alternative to ACE-inhibitors when side effects (such as cough) are encountered. The effects of Ang II are mediated through two receptors; clinically available ARAs block only the AT_1 receptor, but unopposed stimulation of AT_2 receptor by Ang II may be beneficial through AT_2 mediated potentiation of the kinin pathway and increased NO

generation (Liu *et al.*, 1997; Carey *et al.*, 2000). Studies comparing the effects of ACE-inhibitors against ARAs show ARAs to be as effective as, but not superior to long-lasting ACE inhibitors in reducing morbidity and mortality (Dickstein *et al.*, 1995; Lang *et al.*, 1997). In current clinical practice, ARAs may be deemed an alternative to ACE inhibition for treatment of CHF when class specific side effects such as cough (due to increased circulating bradykinin) are encountered, but ACE-inhibitors remain the agents of choice.

1.3.5.3 Vasodilators

The term 'vasodilators' encompasses a wide range of different pharmacological groups, which have widely diverse activities in the peripheral and central circulations. These range from the early α_1-adrenoreceptor antagonists through such groups as the direct acting peripheral vasodilators, nitrates, hydralazine and minoxidil. Also included are the slow calcium antagonists which only dilate the systemic arteriolar resistance vessels; atrial natriuretic peptidase inhibitors with combined arteriolar and venodilator activities, and potassium channel activators with similar combined vasodilator effects.

It has become increasingly common to combine vasodilators such as nitrates or hydralazine with other treatments such as ACE inhibitors. This further enhances cardiac index while reducing pre- and afterload. However, as blood pressure and renal perfusion pressure decline, sodium and water retention may worsen. Thus a delicate balance between optimising systemic hemodynamics and maintaining renal perfusion needs to be achieved (reviewed by Schrier *et al.*, 2000).

1.3.5.4 β-blockers

The primary site of action of β-adrenoreceptor antagonists is thought to be a decrease in renin release form the kidney. β-adrenoreceptor antagonists act by inhibiting the activation of β-adrenoreceptors, thereby preventing the deleterious actions of chronic increased adrenergic stimulation on the failing heart that include an increase in heart

rate, contractility, and atrio-ventricular conduction. Recently, randomised controlled trails have shown the benefits of β-adrenoreceptor antagonists on cardiac function, symptoms, exercise performance and survival. The Second Cardiac Insufficiency Bisoprolol Study (CIBIS-II) found a 34% reduction in mortality and a 20% decrease in the risk of admissions to hospital in those treated with the selective β_1 adrenoreceptor antagonist bisprolol over a mean period of 1.3 years (Lechat *et al.*, 1999). Carvedilol, a non-selective β-adrenoreceptor antagonist, also possessing peripheral vasodilating activity due to α_1 adrenoreacptor antagonism, showed lower mortality rates in two separate studies (Packer *et al.*, 1996; MacMahon *et al.*, 1997). Several other trials of β-adrenoreceptor antagonists in heart failure also showed improvement in survival, cardiac function and symptoms in treated patients (Lechat *et al.*, 1998). However, major side effects such as hypotension, fluid retention, and bradyarrythmias are often related to use of β-adrenoreceptor antagonists. Furthermore, caution is warranted in the initiation and titration of therapy, as symptoms of CHF may transiently worsen.

1.3.5.5 Positive inotropes

Digitalis glycosides have many properties including mild positive inotropy, increased delivery of sodium to the distal tubules leading to suppression of renin secretion from the kidneys, and parasympathetic activation with resultant increased vagal tone. When atrial fibrillation is present in patients with heart failure cardiac glycosides are clearly indicated to lower ventricular response rates. The Digitalis Investigation Group's large study found that digoxin was associated with a sympathomimetic improvement in patients with CHF. But although there was a reduction in the combined end-points of admission, there was no significant improvement in overall survival (Perry *et al.*, 1997).

A major pharmacotherapeutic drawback of digitalis is its narrow dose-response relationship so that the fixed dose selected in order to avoid toxicity often falls short of the effective therapeutic range. Side effects such as cardiac arrhythmias, nausea and neurological complaints are commonly associated with digoxin toxicity.

1.3.6 Endothelin & heart failure

Activation of the ET system, like that of the sympathetic and renin-angiotensin systems, is part of the neural, hormonal and cytokine response of the body to CHF. Patients with CHF have higher plasma ET-1 concentrations (in the vasoactive range 5-40 pmol/L) than healthy individuals (Naruse *et al.*, 1991; McMurray *et al.*, 1992). However, elevated plasma ET-1 is characteristic of severe CHF and not asymptomatic or mild cases, with plasma ET-1 levels being found to increase only in the latter stages of CHF when accompanied by renal failure or pulmonary congestion. Wei *et al.* (1994) reported plasma increases only in patients with moderate (NYHA class III) or severe (NYHA class IV) CHF compared with healthy individuals with asymptomatic (NYHA class I) or mild (NYHA class II) CHF. Measurement of plasma big ET-1 has been recommended as a better marker of CHF, with levels being found to correlate with right atrial pressure, left ventricular ejection fraction and NYHA classification of CHF (Pacher *et al.*, 1993). Furthermore, in a study involving patients with advanced CHF, plasma big ET-1 was strongly related to survival and seemed to predict 1-year mortality better than did haemodynamic variables and concentrations of atrial natriuretic factor, an established prognostic marker of CHF (Pacher *et al.*, 1996).

Although the source of elevated plasma ET levels in established CHF is unclear, at least two mechanisms may be responsible. The first possibility is increased production of ET-1. Studies *in vitro* showed increased ET-1 secretion by the endothelium in the presence of Ang II or AVP (Resink *et al.*, 1990b; Emori *et al.*, 1991). With both AVP and Ang II being elevated in CHF, these may serve as stimuli for ET-1 production (Rubanyi & Polokoff, 1994). In addition to increased production, decreased clearance of ET-1 from the circulation may also occur during CHF. Cavero *et al.* (1990) found that after infusion of the same concentration of ET-1, a 3-fold or greater increase in plasma immunoreactive ET-1 occurred in dogs with CHF when compared to healthy controls indicating, at least in this animal model, clearance of ET-1 was decreased. Although impaired renal function and decreased clearance may be involved in generating high plasma ET-1 levels, increased plasma

levels of big ET-1 (Pacher *et al.*, 1993) suggest that increased synthesis may also contribute.

A number of reports have investigated the expression and distribution of the ET-1 system in human heart failure. ET-1 mRNA expression and immunoreactive ET-1 have been demonstrated in endothelial cells of human cardiac tissue from patients with ischaemic heart disease and idiopathic dilated cardiomyopathy (Plumpton *et al.*, 1996). Giaid *et al.* (1995) showed ET-1 immunoreactivity and preproET-1 mRNA expression localised to vascular and endocardial endothelial cells, as well as to cardiomyocytes in endomyocardial biopsy specimens of transplanted hearts from end-stage heart failure patients. Cardiac myofibres in the immediate vicinity of replacement granulation tissue and fibrosis showed the most intense immunostaining for ET-1 and hybridisation signals for preproET-1 mRNA. However, another human study located ET-1 immunoreactivity of similar intensities in ventricular myocardium from both healthy hearts and from failing hearts of patients with severe CHF (Wei *et al.*, 1994).

Animal models of experimental heart failure have now been designed to further address questions that are difficult to answer in human studies *in vivo*. Most humans with heart failure present in the later stages, thus animal models can be particularly useful as they allow study in the early stages. Advantages include the ability to study the natural history of the syndrome free from the continuous effects of treatment, to carry out experimental procedures not possible in humans, and to test new treatments before their safety is established in humans. Reviews of animal models of heart failure have all generally concluded that no one animal model can mimic the pattern of human heart failure. An ideal model should have a common human counterpart and should closely mimic heart failure in humans. Studies investigating the aetiology of heart failure have reported coronary heart disease (Parameshwar *et al.*, 1992), and ischaemic heart disease (Smith, 1985; Andersson *et al.*, 1993) as major contributors to the development of heart failure.

Many different models have been developed and most are useful in evaluating particular aspects of failure and can provide information not available in the clinic (Doggrell & Brown, 1998). Various models have been used to produce MI in mammals. Coronary ligation has been employed in dogs, but animals that survived the procedure developed small infarcts with only minor haemodynamic abnormalities (Hood *et al.,* 1967). The most widely studied MI model is that produced by coronary artery ligation in the rat (Selye et al., 1960). Animals with large infarcts (>46% of the whole LV) had increased LV diastolic pressure and LV dysfunction (Pfeffer *et al.,* 1979). The popularity of the model also stems from the fact that it is the only model of heart failure caused by IHD (reviewed by Arnolda *et al.,* 1999). Furthermore, responses to treatment in this model have in some instances being confirmed in humans with heart failure, particularly in the case of ACE inhibitors (The CONSENSUS Trial Study Group, 1987; Pfeffer *et al.,* 1988). The rat model of heart failure involving coronary artery occlusion by ligation was used in the investigations of this thesis.

Evidence of a role for ET-1 in the development of experimental CHF is more prominent. In both dog (Margulies *et al.*, 1990) and rat (Sakai *et al.*, 1996a) experimental models of CHF, circulating plasma levels of ET-1 have been shown to be elevated. In CHF induced by pressure overload in the rat, preproET-1 mRNA and ET-1 content were elevated in the hypertrophied left ventricles (Yorikane *et al.*, 1993). Increased preproET-1 mRNA, ET-1 content, and ET receptor density were also demonstrated in the left ventricle 3 weeks after coronary artery ligation in CHF rats (Sakai *et al.*, 1996b), though this group did not attempt to localise the cellular sites of overexpression. Furthermore, myocardial ET-1 mRNA levels were shown to be increased after 1 week in dogs induced with congestive heart failure by rapid ventricular pacing (Margulies *et al.*, 1990).

Questions remain as to how, and more specifically when, and where the myocardial ET-1 system may be upregulated during the development of CHF. ET-1 could contribute towards dysfunction through stimulation of myocardial hypertrophy and ventricular remodelling that occur in response to a myocardial infarction. ET-1 has

been shown to exert hypertrophic effects directly *in vitro* in ventricular cardiomyocytes isolated from hearts of adult rabbits (Mullan *et al.*, 1997). ET-1 also stimulated both α and β-myosin heavy chain gene expression in an isolated neonatal rat myocardial cell culture system (Wang *et al.*, 1992).

ET receptor number and/or sensitivity may play an important role in the actions of ET-1 during the progression of CHF. Cannan *et al.* (1996) showed an attenuated coronary vasoconstrictor response to ET-1 with an enhanced vasoconstrictor response to SRTX S6c, suggesting an alteration in coronary ET receptor sensitivity in a dog experimental model of CHF. Another study found a selective increase in expression of ET-1 and the ET_A receptor subtype in the hypertrophied myocardium of the aorto-venacaval fistula rat (Brown *et al.*, 1995).

Understanding the actions of the ET system during heart failure has been further aided by the development of many compounds that block ET receptors or ET-specific enzymes. The ET_A receptor antagonist BQ-123 and the ECE inhibitor phosphoramidon were shown to cause arterial vasodilatation when infused into the forearm circulation of patients with stable CHF who were already receiving treatment with a loop diuretic and a maximal dosage of ACE inhibitor (Love *et al.*, 1994). In comparison with healthy volunteers, BQ-123 induced vasodilatation tended to be reduced and phosphoramidon-induced vasodilatation increased in patients with CHF, consistent with upregulation of ET_B-mediated vasoconstriction. Furthermore, an interesting study by Sakai *et al.* (1996b) found that administration of the ET_A receptor antagonist BQ-123 (10 days after ligation) to rats for 12 weeks during the development of severe CHF markedly increased survival, while improving left ventricular dysfunction and preventing ventricular remodelling.

However, many studies are now investigating the actions of mixed endothelin antagonists. Infusion of the combined ET_A/ET_B receptor antagonist bosentan for 2 hours (100mg followed 1 hour later by 200mg) in a group of 24 patients with CHF significantly lowered pulmonary arterial blood pressure and vascular resistance and increased the cardiac index (Kaddoura & Poole-Wilson, 1996). Administration of

bosentan to patients with severe CHF also produced sustained systemic, pulmonary and peripheral venous vasodilatation, and improved cardiac performance, without causing reflex tachycardia (Kiowski *et al.*, 1995). These studies have established that ET-1 exerts measurable haemodynamic effects in heart failure. However the haemodynamic effect of antagonists has so far been modest, and improved effects may depend on timing of administration and bioavailability of antagonist used.

Although a significant increase in plasma ET-1 levels has been reported in the early phase of acute MI in patients (Nakamura *et al.*, 1993; Stewart *et al.*, 1991), evidence that ET-1 plays a role in determination of myocardial infarct size following coronary artery occlusion is controversial (Watanabe *et al.*, 1991; Grover *et al.*, 1993; Hide *et al.*, 1995). Antagonist studies have been conducted mainly in acute models of ischaemia and reperfusion. This model, depending on the timing of reperfusion after coronary artery occlusion, produces animal models with varying degrees of myocardial damage. This is different to the model used in the present study, in which irreversible occlusion leads to major myocardial damage in the affected area with blood flow re-entering the infarct, in the initial stages, most probably via collateral flow (Maxwell *et al.*, 1987). Grover *et al.* (1993) indicated a significant reduction in infarct size after administration of the ET_A receptor antagonist BQ-123 into the left circumflex artery, 90 minutes after left coronary artery occlusion and reperfusion. A further study found a monoclonal antibody to ET-1 (AwETN40) reduced infarct size by 38% when administered 5 minutes after induction of coronary artery occlusion for 1 hour (Watanabe *et al.*, 1991). Additionally, another study showed a 0.3nM/kg injection of ET-1 reduced myocardial infarct size by activation of ATP-sensitive potassium channels in a rabbit model of myocardial ischaemia and reperfusion (Hide *et al.*, 1995). Also, intracoronary infusion of the ET_A receptor antagonist FR139317 increased infarct size in the dog, suggesting that endogenous ET-1 may have cardioprotective effects (Velasco *et al.*, 1993). ET-1 has been shown *in vitro* to promote fibroblast proliferation (Takuwa *et al.*, 1989) and increased synthesis of collagen (Guarda *et al.*, 1993); therefore ET-1 may be involved in the processes of scar formation following MI. However, whether myocardial tissue levels of ET-1 change post-MI, and if so, where these changes specifically occur, is not known.

1.4 General Aims of Study

Although evidence of an ET-1 system has been reported in the normal heart (Molenaar *et al.,* 1993; Plumpton *et al.,* 1993), little is known about the specific cellular distribution of ET-1 and its receptors throughout the myocardium. Furthermore, it has been demonstrated that ET-1 levels in the heart increase post-MI, though it is unclear how, when and where these changes occur. Possible mechanisms may involve increased uptake of ET-1 by the heart into the myocardium from circulating plasma, or the myocardium itself may synthesise increased amounts of ET-1 locally due to increased workload and stress induced by a MI. Furthermore, if increased levels of ET-1 peptide are synthesised in the heart early after MI or during development of CHF, it is not clear in which specific cells of the heart these increases are localised.

ET_A/ET_B receptor expression may also change post-MI, though it is unclear which areas of the myocardium and what cells may be specifically affected. There is evidence that antagonist treatment may be important in controlling the ET-1 mediated effects during CHF. It is not known, however, if blocking ET receptors in the early stage after onset of MI is beneficial or detrimental to scar formation.

This thesis set out to investigate the following hypothesis:

1. A local myocardial ET-1 system is present in the healthy hearts of rats.
2. Changes occur in the myocardial ET-1 system that contribute to structural changes in the early stage post-MI and during the progression of CHF.

The main aims of this thesis were therefore to characterise the ET-1 system in healthy hearts of rats, and then to investigate the ET-1 system during the development of CHF following MI with reference to ET-1 peptide, preproET-1, ET_A, and ET_B mRNA expression. Also, a major aim was to investigate any possible correlation between the ET-1 system and myocardial hypertrophy, fibrosis and vascular remodelling during progression of CHF.

With previous studies reporting an increase in plasma ET-1 in the acute phase of MI, a further study was conducted to characterise early stage scar formation following left ventricular MI induced by coronary artery ligation in the rat, with reference to changes in myocardial ET-1 peptide levels. The effect of an ET-1 mixed receptor antagonist on scar formation over the first 2 weeks after MI in the rat was also investigated.

Chapter 2

Methods

This chapter details the experimental techniques that are referred to in more than one results chapter. All experiments were performed using the appropriate level of safety as prescribed in the Control of Substances Hazardous to Health regulations. All animal work was carried out in accordance with the Animals (Scientific Procedures) Act (1986).

2.1 Coronary artery ligation surgery

Male Wistar rats (250±5g, Charles River, UK) were subjected to ligation of the proximal portion of the left coronary artery and infarction of the left ventricular free wall according to the method of Selye *et al.* (1960) and modified by Pfeffer *et al.* (1979). Rats were allowed access to normal rat chow and water ad libitum, for at least 2 days after arrival prior to surgery.

On the day of surgery, the rat was weighed immediately before operation, then anaesthetised by injection of Na pentobarbital (60mg/kg i.p.; Sagatal, Rhone Merieux Ltd., Essex, UK). The front right area of the rat's chest was shaved and cleaned with 70% alcohol. The rat was then placed on a thermostatically controlled heating pad for surgery and intubated (1.5mm diameter plastic cannula, Portex Ltd., UK) via the mouth with the aid of a guide wire.

Mechanical ventilation (room air mixed with 100% O_2) was achieved by use of a small rodent ventilator (Harvard Apparatus Ltd., Kent, UK) at a rate of 60 cycles/minute and a tidal volume of 1ml/100g body weight. Autoclaved instruments were used to cut a 2cm long area of the skin parallel to the direction of the ribs. The underlying muscle layers were gently separated using blunt scissors and held in position by smooth metal clips, revealing the rib cage. A left thoracotomy was performed between the fourth and fifth ribs exposing the inner chest cavity. To avoid damage to the lungs during surgery, the left lung was collapsed by folding a small square of sterilised gauze and using it to cover the lung, which was then gently pushed downward and away from the heart. The pericardium was then cut and the heart gently and rapidly exteriorised by gentle application of pressure to the thorax.

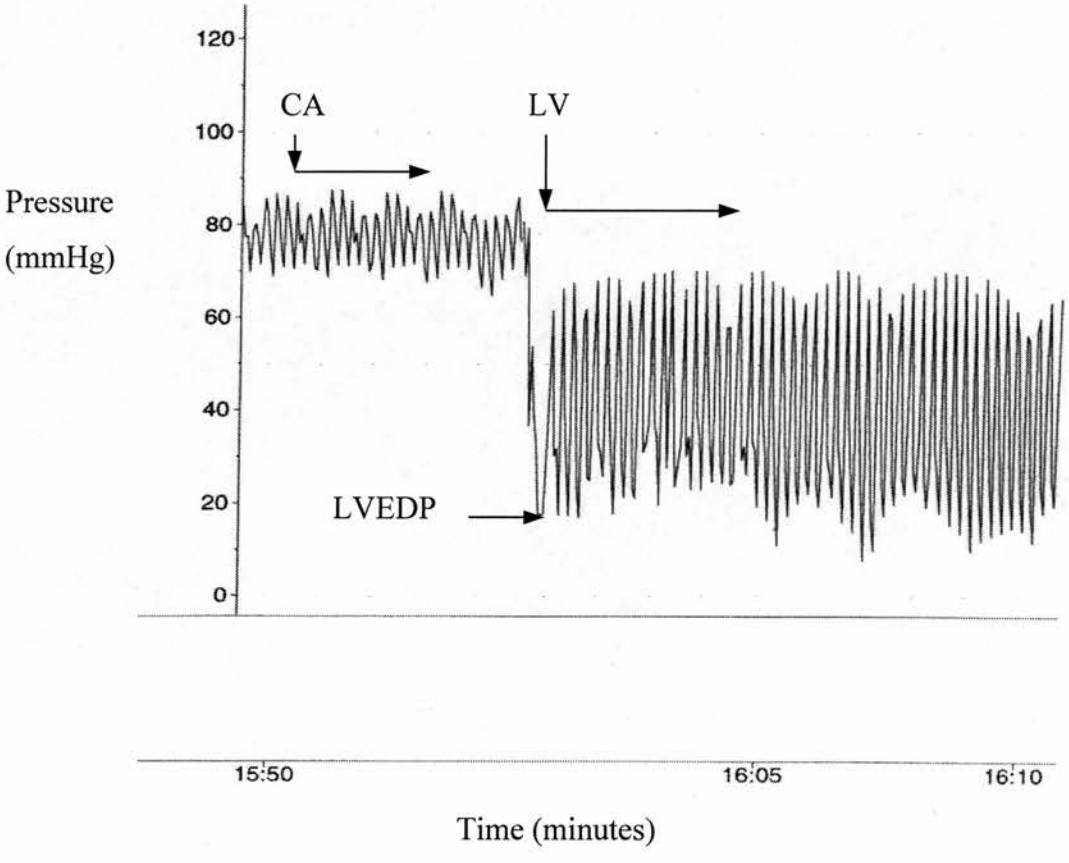
A 10mm round bodied needle (Ethicon® Ltd, Edinburgh, UK) was passed around the proximal left coronary artery. The heart was subsequently returned to its position in the thorax, and the ligature left untied for 10 minutes to permit recovery from initial trauma. The ligature was then tied and the chest incision left open for a further 15 minutes to allow manual manipulation of the heart during any subsequent arrhythmias that occurred after myocardial infarction (MI); usually between 7 and 14 minutes after MI.

Before closing the ribcage, a 0.75mm diameter plastic cannula (Portex) was placed in the chest cavity, and the small square of sterilised gauze retaining the lung removed. The rib cage was then closed by gently squeezing the chest cavity to remove air before inserting 2 stitches of suture (16mm round bodied needle, Ethicon); the remaining muscle was released back over the ribcage and the skin closed with 4 stitches of suture (25mm round bodied needle, Ethicon). The plastic cannula in the chest cavity was connected to a 1ml syringe and used to extract any remaining air from the chest after closure, then gently removed. The rat was then taken off the ventilator and, when spontaneous ventilation was re-established, placed on a heated pad and exposed to 100% O_2 during recovery from anaesthesia. The tracheal cannula was removed upon initial signs of recovery from anaesthesia after which rats were returned to their cages. All rats were given buprenorphine hydrochloride (Vetergesic®, 0.24mg/kg; Redcut & Colman, UK) subcutaneously upon recovery from anaesthetic and again the following morning for analgesia. Sham-operated rats were subjected to the same protocol, except the ligature was not tied, but pulled through under the coronary artery.

2.2 Haemodynamics and tissue sampling

After the allocated period of ligation or sham-operation, the rats were anaesthetised by injection of Na pentobarbital (60mg/kg i.p.). The left carotid artery was located, dissected free of extraneous tissue and cannulated with either a polyethylene fluid filled catheter attached to a pressure transducer (Senso Noras Ltd., Horten, Norway), or a pressure transducer-tipped catheter (Model SPR-407, Millar Instruments, Texas, USA), attached to a MacLab (version 3.4/e) system (AD Instruments, Hastings UK). The catheter was fed via the left carotid artery and aorta into the left ventricle where left ventricular end-diastolic pressure (LVEDP) was measured and analysed on an Apple Mac computer (model LC 475).

Figure 2.1 A sample pressure-transducer reading from within the carotid artery (CA) recording arterial blood pressure, and within the left ventricle (LV) recording left ventricular end-diastolic pressure (LVEDP).



The initial part of the trace (*Figure 2.1*) displays arterial blood pressure in the carotid artery, from which mean arterial blood (MAP) pressure can be measured. As the catheter enters the left ventricle the trace becomes larger, and now indicates left ventricular end systolic pressure. An LVEDP value was measured by calculating the average pressure reading from the troughs of 20 pressure spikes along the LVEDP trace.

Following haemodynamic measurements, the rat was exsanguinated via a needle (1mm diameter, Microlance®, Becton Dickinson, Ireland) placed in the dorsal aorta and an ~8ml blood sample collected into a 10ml syringe pre-rinsed with heparin (100 U/ml final concentration, Multiparin®, CP Pharmaceuticals, Wrexham, UK). The blood was aliquoted into pre-chilled test tubes containing 50µl of 10mmol/l ethylenediaminetetraacetic acid (EDTA; Sigma, UK) and immediately centrifuged (2000*g*, 4°C, 20 minutes). Plasma was aliquoted from each sample and stored at -70°C for future plasma ET-1 and big ET-1 analysis. The heart, lungs and kidneys were excised, rinsed in ice-cooled physiological saline, then individually weighed. The heart was cut apex to base so that each section encompassed both left and right ventricle. All samples were placed in 10% neutral buffered formalin solution (Sigma, UK) for 24hrs fixation. The next day, heart sections were further processed and wax embedded. As the tissue fixative (10% formalin) is in aqueous solution, the water has to be removed in order to embed the tissue in paraffin wax. This was achieved by immersion in increasing strengths of ethanol, and is known as dehydration. Since alcohol and wax are not miscible, the alcohol must be replaced by a wax solvent, and as the majority of wax solvents have the effect of raising the refractive index of tissue, which make them appear clear, this stage has become known as clearing. The heart was finally embedded in wax, while the rest of the organs and tissue were stored at -70°C for future analysis.

2.2.1 LDH (lactate dehydrogenase) assay for confirmation of successful CAL

During periods of hypoxia or induced cellular damage, cells can still produce ATP via anaerobic glycolysis. Glucose is broken down to pyruvate via the following reaction:

$$\textbf{Glucose} + \textbf{NAD}^+ + \textbf{2ADP} \longrightarrow \textbf{Pyruvate} + \textbf{NADH} + \textbf{2ATP}$$

This reaction does not last long as cellular levels of NAD^+ become depleted. However, the cell regenerates NAD^+ by the following reaction:

$$\textbf{Pyruvate} + \textbf{NADH} + \textbf{H}^+ \xrightarrow{\textbf{LDH}} \textbf{Lactate} + \textbf{NAD}^+$$

LDH is an enzyme that catalyses this final reaction of anaerobic glycolysis, namely the reduction of pyruvate to lactate. It is an NADH-linked dehydrogenase and consists of four subunits. There are two kinds of subunit designated M and H. In human skeletal muscle the homogeneous tetramer of M_4 type predominates and in the heart the H_4 tetramer is the predominant form (Apps *et al.,* 1992). Other heterogeneous forms such as M_3H, M_2H_2 and MH_3 occur in blood serum.

A very sensitive clinical test for heart disease is based on the existence of the various isozymic forms of this enzyme. The relative amounts of H_4 and MH_3 isozymes in blood serum increase dramatically after myocardial infarction compared with normal serum. A commercial LDH assay kit (LDH, Boehringer Mannheim, UK) allowed in-lab spectrophotometrical measurement of LDH enzyme levels from plasma samples removed from rats two hours after ligation operation, thereby determining whether a successful ligation had been performed. The basis of the kit utilises the reaction whereby LDH catalyses the reduction of NADH, whose rate of reduction correlates with concentration of LDH present. NADH reduction results in a decrease in absorption at 340nm wavelength, allowing LDH concentration to be indicated over a set time period (Apps *et al.,* 1992).

Briefly, a tail-tip blood sample was collected directly into prechilled test tubes containing EDTA (10mmol/l final concentration), kept on ice and centrifuged at 2000*g* for 20 minutes at 4°C. Plasma was extracted into test tubes and heated to 27°C. From the LDH assay kit, one reagent tablet was added to 3ml of buffer/substrate and also heated to 27°C (end concentration in reagent solution: phosphate buffer: 50mmol/l, pH 7.5; pyruvate: 0.6mmol/l; NADH: 0.18mmol/l). 1.5ml of buffer was then placed in a spectrophotometer set to 340nm wavelength and the reading set to zero. To initiate the reaction, 50µl of plasma was added to the 1.5ml buffer and the spectrophotometer reading taken immediately. Further readings were taken at 1-minute intervals for 3 minutes and the average reading was calculated over the time period. An average reading of at least 3 times greater than that found in plasma from a corresponding sham-op rat indicated a successful ligation.

2.2.2 Measurement of plasma ET-1 / big ET-1.

ET-1 and big ET-1 were measured using an in-house radioimmunoassay (RIA) technique (Hand *et al.,* 1999) and performed with the assistance of Mr. Neil Johnston at the Clinical Pharmacology Unit, Western General Hospital, Edinburgh.

2.2.2.1 Introduction

RIA is a well validated technique used to measure any antigenic component present in a liquid sample. Similar to immunohistochemistry, this technique utilises specific antibodies (Abs) raised against the antigenic substance in question, followed by the addition of radiolabelled antigen (Ag). The radiolabelled, precipitated Ag/Ab complexes are then measured in a gamma counter. Therefore, the higher the concentrations of endogenous antigen, the lower the amount of bound radiolabelled antigen and the subsequent radiation counts. For this thesis, both ET-1 and big ET-1 levels were measured in plasma samples from rats undergoing either coronary artery ligation surgery or sham-operation to ascertain whether there was increased circulating production of the peptide. Increased circulating levels of ET-1 and big

ET-1 have been demonstrated in both human and animal studies (see section 1.3.6). However, it is important to delineate between the two forms of the peptide, since it may help in demonstrating whether there is an upregulation of the production of ET, or reduced clearance and elimination of the mature peptide, in CHF. In order to answer this question, primary, polyclonal antibodies raised in rabbits against human ET-1 and big ET-1 (Peninsula Labs, USA) were used. These antibodies have a 100% reactivity with rat ET-1 and big ET-1. Furthermore, The ET-1 Ab had a cross reactivity with big ET-1 of 10%, and the big ET-1 Ab had a cross reactivity with ET-1 of 8 %.

There are two main components to the measurement of ET-1 or big ET-1 from the plasma samples in the RIA technique. These are, the extraction of either ET-1 or big ET-1 from the sample, and subsequent RIA of the extracts.

2.2.2.2 Extraction technique

An ~8ml blood sample was collected as previously described (Section 2.2). ET-1 and big ET-1 had to be extracted from the plasma samples due to the extremely low circulating levels of these peptides. The peptides were extracted using C_{18} 200mg Bond Elut Extraction Cartridges (Varian Sample Preparation Products, Switzerland). These cartridges are internally coated with a silica sorbent that consists of an octadecyl carbon matrix with high affinity for peptide molecules containing polar groups, such as ET-1 and big ET-1.

Prior to extraction of the sample, the extraction columns had to be activated to obtain the correct conditions for the extraction of either ET-1 or big ET-1. For ET-1 and big ET-1 extraction, 3ml of 100% methanol (BDH-Merck, UK) was added to the column and allowed to flow through. The columns were washed with deionised water, and then brought to pH 5.5, by the addition of 2ml of 10% acetic acid (BDH-Merck, UK). After thawing, the plasma samples were acidified with 20% acetic acid (1:1 v/v; 2.5ml:2.5ml), added to the column for extraction and left to flow through by gravity. The column was washed with 2ml of 10% acetic acid to remove any

unwanted proteins, followed by 3ml of ethyl acetate (BDH-Merck, UK) to remove any unwanted lipids, from the column. To elute the ET-1 fraction from the extraction columns, collection tubes were placed under the extraction columns, and 1.5ml of elution buffer (80% methanol / 20% 0.05M ammonium bicarbonate solution; BDH-Merck, UK) was added to the columns and allowed to flow through by gravity.

Following the collection of the eluted ET-1 or big ET-1 extracts, the elutes were dried down under nitrogen (British Oxygen Company, UK) in a water bath at 37°C. The dried elutes were reconstituted with 0.25ml assay buffer, and stored at –40°C until assay (generally the next day). Previous validation studies in the laboratory have demonstrated that the extraction/recovery rate is 89% for the ET-1 protocol and 91% for the big ET-1 methodology (Hand *et al.,* 1999).

2.2.2.3 Standard curves

The standard concentrations used in the ET-1 and big ET-1 RIAs were as follows. The stock concentration of ET-1 (Peninsula Labs, USA) was 64pg/ml. Serial 1:1 dilutions using assay buffer were performed, giving concentrations of 32pg/ml (S32), 16pg/ml (S16), 8pg/ml (S8), 4pg/ml (S4), 2pg/ml (S2) and 1pg/ml (S1). The stock concentration of big ET-1 (Peninsula Laboratories, UK) was 128pg/ml (S_{128}), as for the ET-1 standard curve, serial 1:1 dilutions using assay buffer gave standards of S_{64}, S_{32}, S_{16}, S_8, S_4, S_2, S_1 and $S_{0.5}$.

Duplicates of 100µl of each sample and standard were assayed. Furthermore, 3 other control tubes were made up and assayed. These were the 'Blank', which contained only assay buffer solution, ^{125}I-ET-1 or ^{125}I-big ET-1 and Amerlex™ donkey anti-rabbit antibody (Amersham Life Sciences Ltd., UK); a 'Reference' tube which contained assay buffer solution, primary Ab, ^{125}I-ET-1 or ^{125}I-big ET-1; and Amerlex™ and the 'Total Counts' tube which consisted of assay buffer and ^{125}I-ET-1 or ^{125}I-big ET-1.

2.2.2.4 ET-1 RIA protocol

After thawing of the ET-1 extracts, 100µl of the primary anti-human ET-1 Ab (1:20,000; Peninsula Laboratories, UK) was added to 100µl of sample, standard and reference tube (but not the blank or total tubes), vortexed and left to incubate at room temperature for 4 hours. The ET-1 Ab had previously been diluted 1:20 in 0. 1% Triton-X (BDH-Merck) in assay buffer solution. Following incubation, 100µl of ^{125}I-ET-1, which is diluted to contain 7500 cpm, was added to the samples, standards, reference, blank and total counts tubes, vortexed and allowed to incubate overnight at 4°C.

In order to separate out the Ag/Ab complexes from the unbound ^{125}I-ET-1, 200µl of Amerlex™ was added to all tubes, except the total tube, vortexed and incubated for 30 minutes at room temperature. Amerlex™ is a separation reagent, which consists of magnetic polymer beads coated with donkey, anti-rabbit IgG, which binds to the bound Ag/Ab complex. The separation of the free and bound fractions were carried out using a magnetic separator and centrifugation at 4°C allowing the beads to migrate to the base of the tube, taking with them any bound, labelled complex. The unwanted supernatant containing any unbound ^{125}I-ET-1, as well as any other substances present in the sample, was aspirated off and the resulting pellets were counted for radioactivity in the Gamma counter (Wallac, Finland). The standard curves were plotted using an automatic sigmoid plot line (spline) programme within the counter, and the levels of ET-1 present in each sample calculated from the standard curve.

2.2.2.5 Big ET-1 RIA protocol

A similar methodology to the ET-1 RIA was used for measurement of big ET-1 levels. However, 100µl of rabbit anti-human big ET-1 Ab (Peninsula Laboratories, UK) was added to the relevant tubes and samples and left to incubate overnight at room temperature. 100µl of ^{125}I-big ET-1 was added on the second day of the assay.

After overnight incubation at 4°C, 200µl of Amerlex™ was added to each tube (except the Total), and the rest of the protocol performed as for the ET-1 RIA.

2.2.3 General Staining

2.2.3.1 van Gieson's collagen stain

Wax embedded hearts were sectioned at 3µm and floated out on a water bath set at 48°C. Sections were then placed on TESPA (3-Aminopropyltriethoxy-saline; Sigma, UK) - coated slides (see Appendix) and allowed to adhere overnight in an oven at 37°C. For collagen staining, sections were initially dewaxed through xylene and rehydrated through 100, 90 and 70% alcohol solutions before being placed in water for 15 minutes. Sections were then dipped in Celestine Blue nuclear stain for 2 minutes, immersed in van Gieson's stain for 3 minutes, then dehydrated through 70, 90 and 100% alcohol solutions and xylene before being mounted in DePeX mounting medium (BDH Laboratory Supplies, UK).

2.2.3.2 Haematoxylin and eosin stain

3µm tissue sections were dewaxed and rehydrated as described above. Sections were then immersed in haematoxylin (Harris', BDH, UK) for 3 minutes, and rinsed briefly in distilled water before being immersed in 1% eosin (BDH, UK) for 5 minutes. Finally, sections were dehydrated through 70, 90 and 100% alcohol solutions and xylene before being mounted in DePeX mounting medium.

2.2.3.3 Neutrophil stain

Neutrophils were detected in myocardial granulation tissue using a diagnostic kit that highlights Naphthol AS-D Chloroacetate Esterase (Sigma, UK), an enzyme that is present in, and usually considered specific for, cells of granulocytic lineage. Briefly, 3µm tissue sections were dewaxed and rehydrated as described above. Sections were

then incubated with napthol AS-D chloroacetate in the presence of freshly formed diazonium salt protected from light for 30 minutes at 37°C. Enzymatic hydrolysis of ester linkages liberates free napthol compounds. These couple with the diazonium salt, forming highly colored (purple) deposits at sites of enzymatic activity. Finally, sections were covered with mounting medium (Aquamount Improved, BDH, UK) and placed in coverslips.

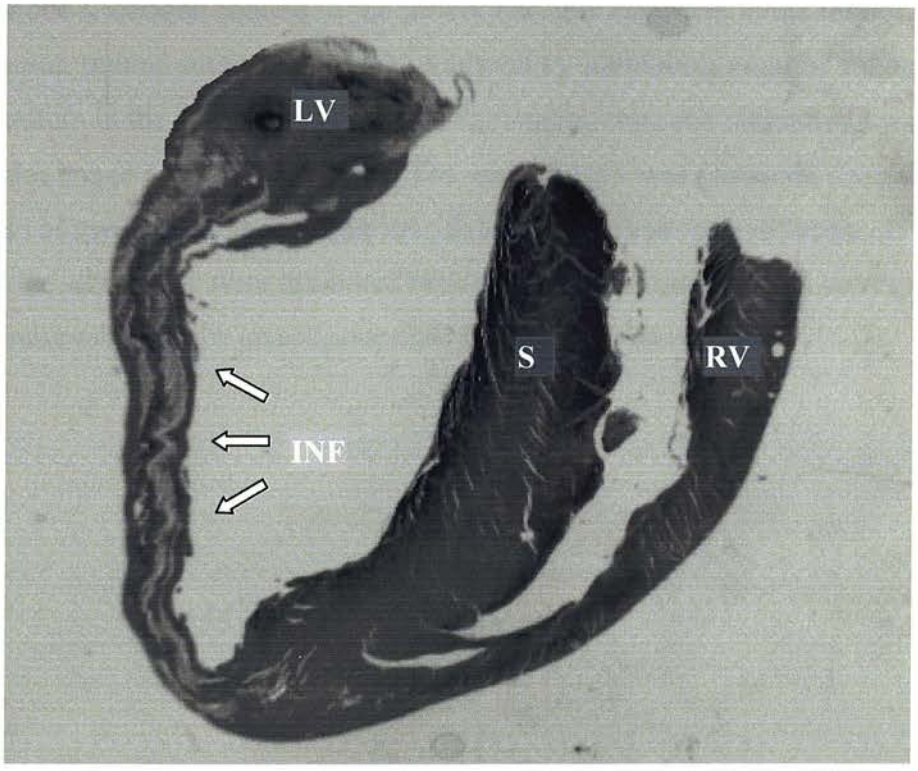
2.2.3.4 Apoptosis Assay

Apoptotic cells were detected using a DeadEnd™ Colometric Apoptosis Detection System (Promega, UK), which end-labels the fragmented DNA of apoptotic cells using a modified *in situ* nick end-labelling (TUNEL) assay. Biotinylated nucleotide was incorporated at the 3'-OH DNA ends using the enzyme Terminal deoxynucleotidyl Transferase (TdT). Horseradish-peroxidase-labelled strepavidin was then bound to these biotinylated nucleotides, which were detected using the peroxidase substrate hydrogen peroxide and the stable chromagen diaminobenzidine (DAB). Apoptotic nuclei appeared brown. The procedure was carried out with the assistance of Mr. David O' Regan.

2.2.4 Measurement of infarct parameters

Sections from hearts stained with van Gieson's collagen stain were used to measure different parameters of the infarct including infarct size, thickness and myocardial / collagen ratio of the infarct. Infarct size was measured as previously described by Mulder *et al.* (1997). Briefly, sections were placed under a CCD video camera module (Sony, UK) attached to a microscope with a x20 lens, and an image of the heart brought up on computer as shown below (*Figure 2.2*).

Figure 2.2 Scanned image of a longitudinal section of heart, which is used for measuring infarct parameters. Key: LV, left ventricle; RV, right ventricle, S, septum; INF, area of infarction.



The endocardial and epicardial circumferences of the infarcted tissue and of the left ventricle were determined with image analysis software (Zeiss Kontron 300 image analysis package, Image Associates, Thame, UK). Infarct size was calculated as:

$$\frac{\textbf{[endocardial +epicardial circumference of the infarcted free left ventricle (mm)]}}{\textbf{[endocardial +epicardial circumference of the whole free left ventricle (mm)]}}$$

and expressed as a percentage. Infarct thickness was measured by randomly selecting 4 points along the length of the infarct and calculating an average value in mm.

Myocardial / collagen content of the infarct was also measured in the infarct wall of van Gieson stained sections. This was achieved by identifying areas of collagen and myocardium in the infarct zone using the light microscope and measuring these areas using the image analysis equipment. The area of each was measured (in mm^2) and the ratio of myocardium to collagen calculated. All sections from different MI groups within the same study were examined blinded, with heart numbers and corresponding group reserved from the investigator until the analysis was complete.

2.3 Antagonist administration protocol

An experimental protocol was designed to administer an endothelin ET_A/ET_B receptor antagonist, A-182086 (kind gift from Abbott Laboratories, Illinois, USA) to groups of rats that had undergone coronary artery ligation or sham-operation. The following initial experiments were performed to investigate if rats would drink water containing A-182086. Also, experiments were performed to show whether rats would assimilate the antagonist and, finally, if a concentration of 15mg/kg rat/day (recommended by Abbott Laboratories) was sufficient to block both ET_A and ET_B dependent responses to ET-1.

2.3.1 Drinking Water Pilot Study

Initial studies indicated that a 250g rat drinks on average 35 ml of water per day (*Table 2.1*).

Table 2.1 Average volume of water consumed by a rat per day.

Rat No.	weight (g)	Volume H_2O given	Overall amount drunk (ml) day 1	day 2	Average volume consumed (ml)	Average volume/ weight
1	250	50 ml	34	34	34	0.136
2	232	50 ml	34	33	33.5	0.144
3	212	50 ml	28	28	28	0.132
4	221	50 ml	29	28	28.5	0.129
					Average	0.135
					Standard Error	0.003
					n	4

Therefore, a rat of Xg weight will drink 0.135X ml/day (35ml/250g = 0.135ml/g rat). Rats were to receive 15mg/kg/day of antagonist; therefore a 250g rat receives 3.75mg/day. Antagonist was subsequently added to the drinking water at a concentration of: 3.75mg day^{-1}/35ml day^{-1} = 0.1071mg/ml.

Rats were individually caged and allowed free access to the water throughout the study, which lasted either 2 or 7 days. After each 24-hour period, the amount of

water drunk from each bottle was measured to allow assessment of water, and therefore antagonist, consumption per rat.

2.3.1.1 Protocol to test uptake of A-182086 into rat and effective blocking of receptors.

After an allocated time of 2 or 7 days, rats (n=2 per group) were anaesthetised by injection of Na pentobarbital (60mg/kg i.p.). The left carotid artery was located, dissected free of extraneous tissue and cannulated with a pressure transducer-tipped catheter (Model SPR-407, size 2F, Millar®, Texas, USA), attached to a MacLab (version 3.4/e) system (AD Instruments, Hastings UK). A further 0.75mm diameter cannula was inserted into the right jugular vein and used to administer 0.05ml bolus doses of endothelin-1 (ET-1) at concentrations of 0.1nM, 0.3nM and 1nM (made up in 0.9% saline). Changes in arterial blood pressure were recorded on an Apple Mac computer (model LC 475) and analysed for the effect of ET receptor antagonism.

2.3.1.2 Results from drinking water pilot study

Those rats not exposed to A-182086 (control, n=2) produced a dose response to ET-1 which consisted of an initial ET_B receptor-mediated vasodilation followed by a prolonged ET_A receptor-mediated vasoconstriction (*Figure 2.3.*). Both vasodilator and vasoconstrictor responses were more pronounced at the higher ET-1 concentrations. In rats allowed free access to drinking water containing A-182086 (15mg/kg) the ET_B receptor-mediated vasodilation response was substantially reduced after 7 days (*Figure 2.4*), but only partially blocked after 2 days (not shown). However, ET_A mediated vasoconstriction was not affected at either time point.

These results suggested that a higher concentration of antagonist would be needed to block both receptors at both time points, therefore a concentration of 30mg/kg was used as indicated by Abbott as being in the high dose range for blocking both ET

Figure 2.3 Dose responses to ET-1 (0.1, 0.3 and 1.0nM i.v.) in an anaesthetised control rat. Key: MAP, mean arterial pressure.

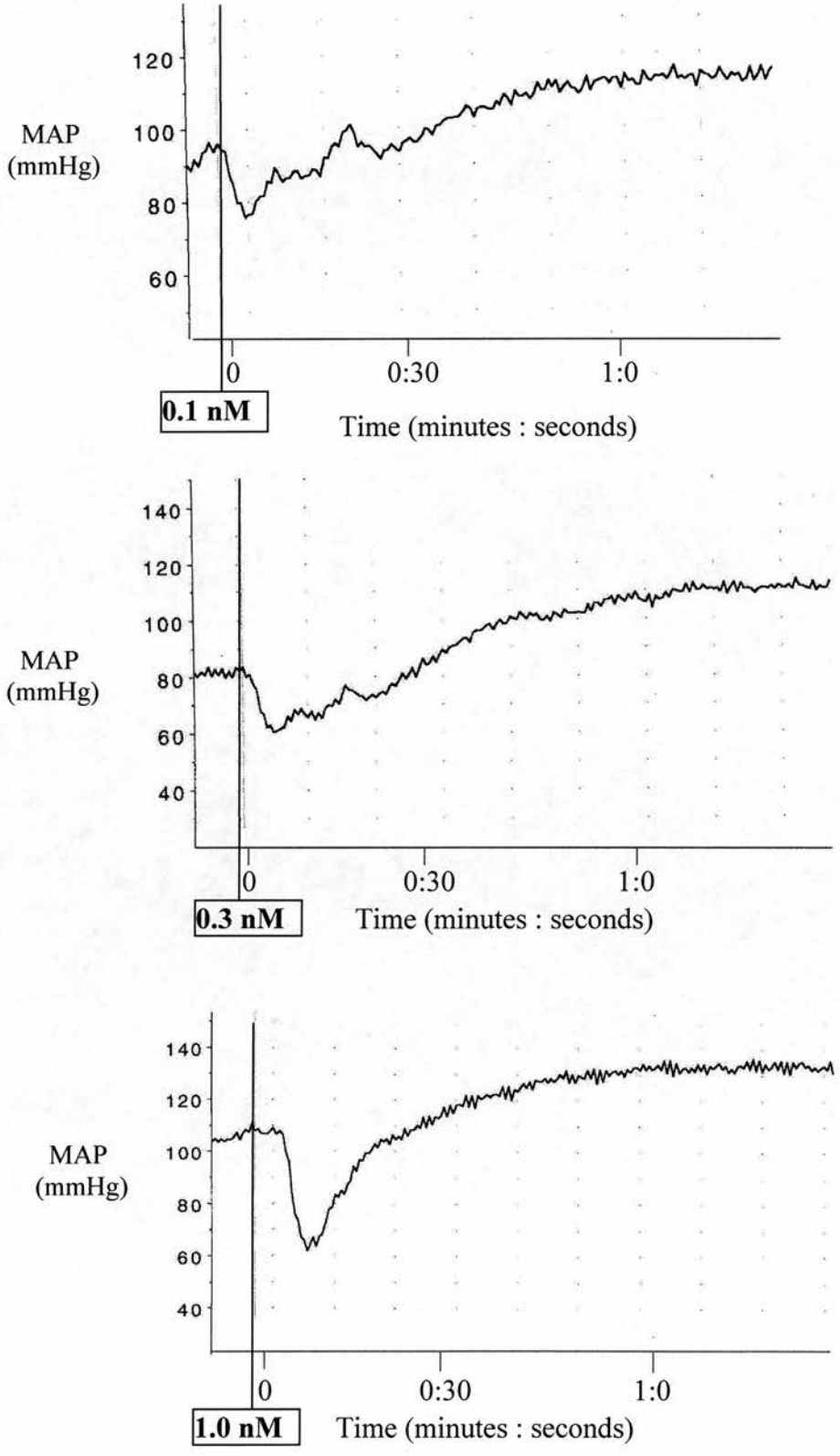
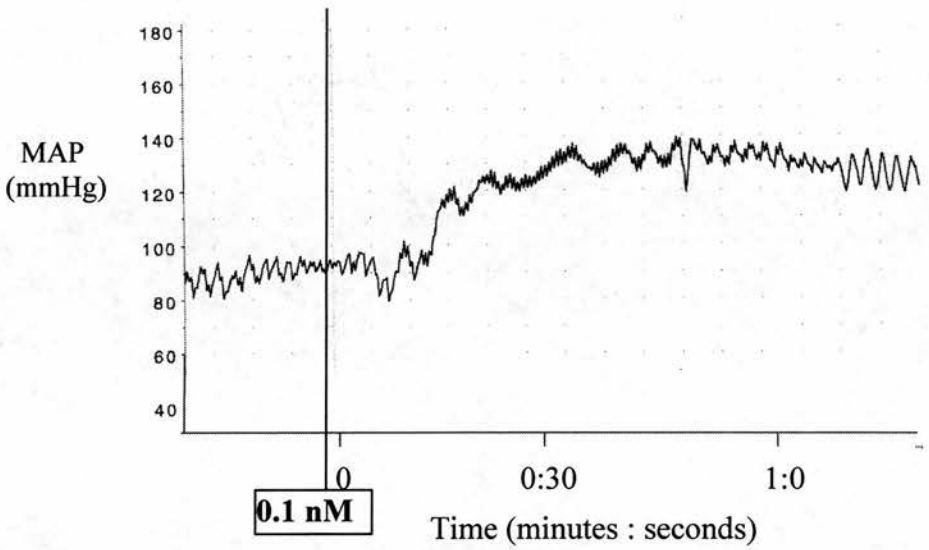


Figure 2.4 Response to 0.1nM ET-1 in an anaesthetised rat after exposure to A-182086 in drinking water for 7 days.



receptor types in rats. Also, due to the relative difficulty in measuring and assessing drinking levels (and therefore antagonist consumption), a simpler protocol was designed to assess the feasibility of drug administration in food.

2.3.2 Milupa Pilot Study

This protocol involved supplementing the normal diet with a mixed cereal feed (Milupa® mixed cereal baby feed, Seven Seas, UK) administered as a paste (5g + 9ml water) containing a precise amount of drug calculated for each rat. The rats were maintained on a restricted diet throughout the experiment to ensure that all food given to them, and therefore the complete drug dose, was ingested.

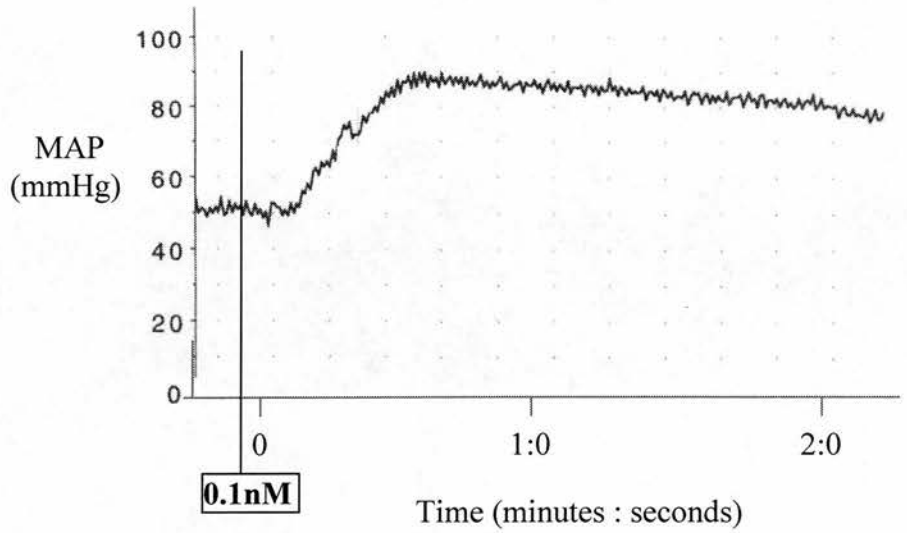
Rats were brought into the animal house and left for 2 days (day 0, day 1) to recover from travel prior to surgery and fed under normal animal in-house conditions. On day 2, rats were weighed, then started on a restricted diet of 5g Milupa for the next 24 hrs. On day 3, rats were re-weighed (should be between 80-90% of initial weight), then given 5g Milupa with added drug and 5g of standard pellet chow (total food weight =10g). The drug was administered at a concentration of 30mg/kg/day to each individually caged rat in the 2 groups and was added daily to the Milupa only e.g. (a 250g rat would receive 7.5mg of A-182086 in the Milupa).

From day 4 onwards, rats were re-weighed, and given the same drug/Milupa dose. If a rat had gained or lost more than 4g in weight over previous 24 hrs, then the amount of standard pellet chow added was adjusted by decreasing or increasing the amount given by 0.5g accordingly. This procedure was continued until the rat had finished the dosing time cycle. Time cycles of 2 and 7 days were used for this pilot experiment, after which rats were sacrificed and underwent a similar ET-1 dose response protocol to those rats undergoing the drinking water study.

2.3.2.1 Results from Milupa pilot study.

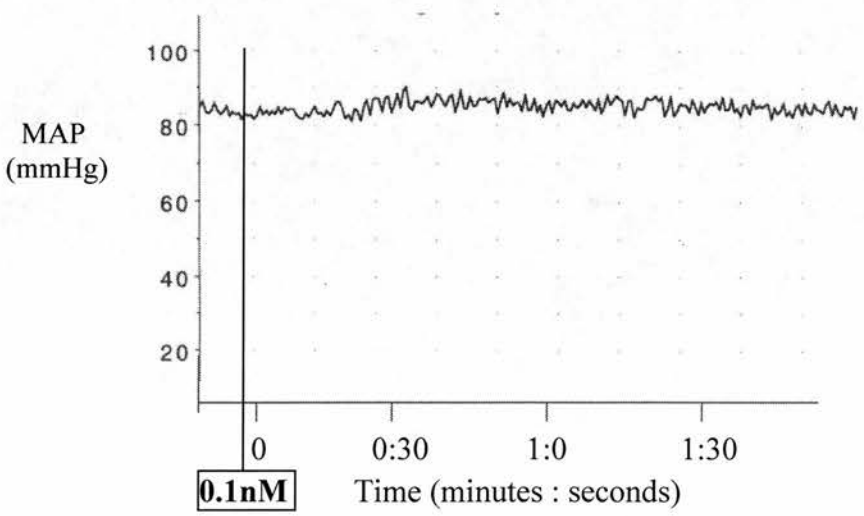
An ET-1 dose response in rats (n=2) after 2 days antagonist (30mg/kg/day) treatment in food showed ET_B-mediated vasodilation to be completely blocked, but ET_A-mediated vasoconstriction only partially blocked (*Figure 2.5; 0.1nM response shown only*).

Figure 2.5 The response to 0.1nM ET-1 (i.v.) in an anaesthetised rat fed Milupa plus A-182086 for 2 days.



The lack of complete antagonism of the ET_A receptor-mediated response may have been due to the length of time between final drug consumption (28-30 hours) and dose response experiment (the following day.). Therefore it was suggested that rats from both groups would receive a further supplement of Milupa plus antagonist on the morning of the day of sacrifice. Dose response experiments were then carried out approximately 3 hours after Milupa plus antagonist consumption. Responses to both ET_A and ET_B receptors were now shown to be completely blocked (*Figure 2.6*).

Figure 2.6 The response to 0.1nM ET-1 (i.v.) in an anaesthetised rat from the 2 day antagonist group also fed on the morning of sacrifice.



Therefore, these pilot studies indicated that the most efficient method of drug administration was in food with an antagonist concentration of 30mg/kg/day showing effective blockade of both ET_A and ET_B-mediated responses. Also, all animals undergoing operation and antagonist treatment would be fed their first supplement of A-182086 upon recovery after operation and subsequent doses each morning up until the day of sacrifice.

2.3.3 Protocol used for rats undergoing coronary artery ligation (CAL) plus antagonist administration study

The following protocol was therefore used for administering A-182086 to the groups of rats used in this experiment. Initially, rats (200-225g) were brought into the animal house and fed under normal animal house conditions and given free access to water. After 2 days, the restricted diet protocol was followed to maintain individual rat weights at 250±5*g* throughout the protocol, thereby allowing a constant drug dose of 7.5mg/day to be administered.

On the day of surgery, CAL was performed as described previously and the rat allowed to recover. Upon recovery, approximately 2-3 hrs after ligation, a tail-tip blood sample was taken for measurement of plasma LDH levels using a commercial assay kit (LDH, Boehringer Mannheim, UK). This was performed to assess the level of damage to the heart and therefore an indication of a successful ligation. Rats that had an LDH level at least 3-fold greater than sham-op levels were deemed to have a successful ligation and were continued in the study (see section 2.3.1 for more detailed discussion). Both sham-op and CAL rats were then randomly allocated to receive antagonist with Milupa 'paste'. Rats undergoing antagonist treatment were therefore given 5g Milupa paste with added drug upon recovery. Those rats not undergoing antagonist treatment received 5g Milupa paste only.

The following morning (day 1) rats were again weighed, then given 5g Milupa (with drug mixed in for those rats undergoing antagonist treatment) and 5g of standard pellet chow (total food weight =10g). Therefore, rats undergoing antagonist

treatment received 2 drug doses within the first 24 hrs after CAL. The original schedule of feeding once every 24 hours was continued again on day 2 (with the weight of chow fixed accordingly with changing weight of the rat) and continued for a period of 2, 7 or 14 days whereupon the rats were sacrificed. For each group of CAL and sham-op rats undergoing antagonist treatment, there was an equal group, which went without. Overall, there were 12 groups in this study (*Table 2.2*).

Table 2.2 Number of animals allocated to each of the 12 groups in the study. Rats were sacrificed at 2, 7 or 14 days after sham-operation (Sham-op) or coronary artery ligation (CAL). A = antagonist (30mg/kg rat/day in food).

Group	**Sham-op**	**CAL**	**Sham-op + A**	**CAL+A**	Total
2 day	8	8	6	6	28
7 day	8	8	7	7	30
14 day	8	8	7	8	31
Total	24	24	20	21	89

2.4 Immunohistochemistry

2.4.1 Background to Immunohistochemistry

Immunohistochemistry (IHC) is a technique for identifying cellular or tissue constituents (antigens) by means of antigen-antibody interactions, the site of the antibody binding being identified either by direct labelling of the antibody, or by use of a secondary labelling method. Antibodies are formed in the humoral immune system by plasma cells. Immunoglobulin (Ig) G, the commonest and most frequently used antibody for immunohistochemistry, is composed of two pairs of light and heavy polypeptide chains linked by disulphide bonds to form a Y shaped structure. The terminal regions of each arm vary in amino-acid sequence and this variability provides specificity for a particular epitope and enables the antibody to bind specifically to the antigen against which it was raised.

The primary reagents in IHC are antibodies (polyclonal and monoclonal) and lectins. Immunising a host animal with a purified specific molecule (immunogen) bearing the antigen of interest produces polyclonal antibodies. It is likely that the animal will produce many clones of plasma cells with each clone producing an antibody with a slightly different specificity to the variety of epitopes present on the immunogen (De Mey & Moeremans, 1986). Some of these antibodies may cross-react with other molecules and will need to be removed by absorption with the appropriate antigen.

In 1975, Kohler and Milstein developed the procedure for generating monoclonal antibodies. This technique involves fusing activated B cells (source of antibodies) with myeloma cells in culture. The result is a clone of antibody producing cells, which continue to grow and divide in culture. By careful screening, hybrids producing antibodies of interest without cross-reactivity to other molecules can be chosen for further cloning. The end result is a supply of one pure antibody with known specificity. The advantage of monoclonal antibodies is their absolute specificity for a single sequence or 'epitope' on the antigen molecule.

Lectins are sugar-binding proteins or glycoproteins of non-immune origin which are derived from both plants and animals. Most lectins studied are multimeric, consisting of non-covalently associated subunits. It is this multimeric structure which gives lectins their ability to agglutinate cells or form precipitates with glycoconjugates in a manner similar to antigen-antibody interactions. Since virtually all biological membranes and cell walls contain glycoconjugates, all living organisms can be studied with lectins. They can be labelled in similar ways to antibodies or identified by using lectin-specific antibodies as secondary reagents.

Enzymes are the most widely used labels in immunohistochemistry and incubation with a chromogen using a standard histochemical method produces a stable, coloured reaction end product suitable for light microscopy. The two most common enzyme labelled conjugate systems used to detect antigens are the peroxidase method and the alkaline phosphatase method. Horseradish peroxidase is the most widely used enzyme and in combination with the most favoured chromogen, i.e. 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma, UK), it yields a crisp, insoluble, stable, dark brown end product. Alkaline phosphatase is the most widely used alternative enzyme tracer to peroxidase. New fuschin substrate produces a permanent insoluble red product at sites of alkaline phosphatase activity.

The method used for antigen detection in this thesis is the indirect antigen technique. Here, the primary unconjugated antibody is allowed to bind to the antigen in the tissue section. A second tracer-conjugated antibody, raised in another animal host and specific for the animal and immunoglobulin class of the primary antibody, is applied to the section and allowed to bind to the primary antibody. The complex that forms can be visualised by incubation of the section in an appropriate substrate.

Table 2.3 List of Antigens, Antibodies (with concentrations used) and Substrates in IHC experiments.

Antigen	**Endothelial / inflammatory cells**	**TGF-β_1**	**ET-1**	**ET-1**	**β-MHC**	**ET_B**	**ERK**
Primary Antibody	GSL I (1:400)	Chicken anti-TGF-β_1† (1:40)	Mouse anti-ET-1* (1:300)	Rabbit anti-mature ET-1* (1:20)	Mouse anti-β-MHC (1:200)	Sheep anti-ET_B† (1:300)	Mouse anti-ERK (1:500)
Supplier	Vector Laboratories, UK	R&D Systems Ltd., UK	Biodesign, UK	Anthony Davenport University of Cambridge, UK	Biogenesis Ltd., UK	Calbiochem, UK	Transduction Lab., UK
Secondary Antibody	Goat anti-GSL I (1:200)	Rabbit anti-Chicken AP IgG (1:60)	Goat anti-Mouse AP IgG (1:60)	Goat anti-Rabbit AP IgG (1:60)	Goat anti-Mouse Biotinylated IgG (1:150)	Donkey anti-Sheep Biotinylated IgG (1:150)	Goat anti-Mouse Biotinylated IgG (1:150)
Supplier	Vector Laboratories, UK	Sigma Ltd., UK	Dako Ltd., UK	Vector Laboratories,UK	Vector Laboratories, UK	Vector Laboratories,UK	Vector Lab., UK
Tertiary Antibody	Rabbit anti-Goat AP IgG (1:60)						
Supplier	Vector Laboratories, UK						
Substrate	New Fuschin	New Fuschin	New Fuschin	New Fuschin	DAB	DAB	DAB
Supplier	Dako Ltd., UK	Dako Ltd., UK	Dako Ltd., UK	Dako Ltd., UK	Sigma Ltd., UK	Sigma Ltd., UK	Sigma Ltd., UK

Key: GSL, *Griffonia Simplicifolia Lectin*; * monoclonal antibody; † polyclonal antibody; AP, alkaline phosphatase

2.4.2 IHC Alkaline phosphatase protocol

Immunohistochemistry for detection of ET-1, TGF-β_1 and endothelial cells was performed on sections of cardiac ventricular tissue taken from the CAL and sham-op groups of rats using the alkaline phosphatase method of antibody detection.

For detection of inflammatory cells, immunohistochemistry using different antibodies directed at specific inflammatory cell surface markers such as CD 11b (Serotec Ltd., UK) or MAC 387 (NeoMarkers, CA, USA) were performed. Both the alkaline phosphatase method and the peroxidase method were utilised, though cell specific staining could not be accomplished. Also, changes in antibody and serum blocking concentration or incubation periods could not improve cell specific staining. However, the endothelial cell marker GSL I was also found to be an excellent marker of inflammatory cells (which were easily distinguishable from endothelial cells by their larger cell size and defined nucleus). Therefore, though GSL I was initially acquired as a marker for endothelial cells, it was also used to highlight inflammatory cells via the alkaline phosphatase method of antibody detection.

Wax embedded hearts were sectioned at 3μm and floated out on a water bath set at 48°C. Sections were then placed on TESPA (3-Aminopropyltriethoxy-saline; Sigma, UK) -coated slides (see Appendix) and allowed to adhere overnight in an oven at 37°C. The following day, sections were dewaxed in xylene, rehydrated in 100, 90 and 70% alcohol solutions and placed in water for 15 minutes before being washed in pH 7.6 phosphate buffered saline (PBS) buffer (see Appendix) for 5 minutes.

During the process of formalin fixation, antigenic sites may become masked and treating sections with proteolytic enzymes may restore the immunoreactivity of masked antigens. Enzymatic pre-treatment of sections is reported to enhance not only immunohistochemical staining but also to improve the reliability and sensitivity of immunocytochemical methods. Therefore, sections were primarily incubated in pH 7.8 tris-buffered saline (TBS; see Appendix) for 15 minutes at 37°C before being

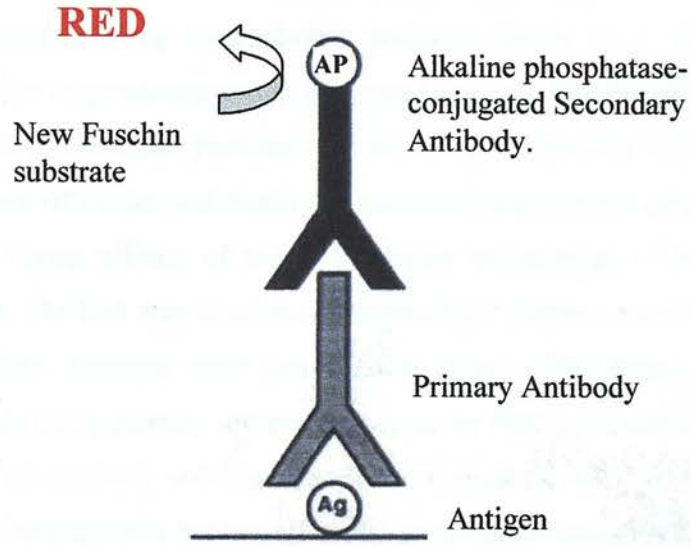
treated with 0.1% trypsin (made up in TBS) for 45 minutes at 37°C, these incubation conditions giving optimum enzyme interaction.

The main cause of non-specific background staining is a non-immunological binding of the specific immune sera by hydrophobic and electrostatic forces to certain sites within the tissue sections. Blocking those sites that show non-specific affinity for immunoglobulins can reduce background staining. This can be achieved by incubating the sections in an immunoglobulin, which will not react or interfere with the primary specific antiserum. The section is generally incubated in normal whole serum from the species in which the secondary antibody is raised. Sections were therefore treated with 1% serum (goat/rabbit serum diluted in PBS) for 30 minutes at room temperature, then incubated in either 1/300 mouse anti-ET-1, 1/40 chicken anti-TGF-β_1, or 1/500 *Griffonia Simplificolia Lectin I* (GSL I; for endothelial cells and inflammatory cells) primary antibody overnight at 4°C. For negative control sections, the primary antibody was replaced with an identical antibody, which lacked the specific antigen recognition site.

The following morning sections were rinsed in PBS, incubated with 1/60 alkaline phosphatase-conjugated goat anti-mouse, 1/60 rabbit anti-chicken IgG secondary antibody, or 1/200 goat anti GSL I (for endothelial cells) for 30 minutes. Sections were then washed again in PBS for 15 minutes before addition of a tertiary antibody (1/60 alkaline phosphatase conjugated rabbit anti-goat IgG) for GSL I. ET-1, TGF-β_1 and endothelial cell immunoreactivity was detected using new fuschin substrate (Dako Ltd., UK). A red product was precipitated at sites of alkaline phosphatase enzyme interaction 10-15 minutes after application of substrate (*Figure 2.7*). All antibody concentrations and incubation times were optimised in-lab from values recommended by the specific antibody supplier.

Sections were rinsed in distilled water for 5 minutes and secondary stained for 3 minutes using haematoxylin (Harris', BDH, UK) that highlights nuclei in purple. Finally, sections were covered with mounting medium (Aquamount Improved, BDH, UK) and placed on coverslips.

Figure 2.7 Diagram of Alkaline Phosphatase antibody binding.



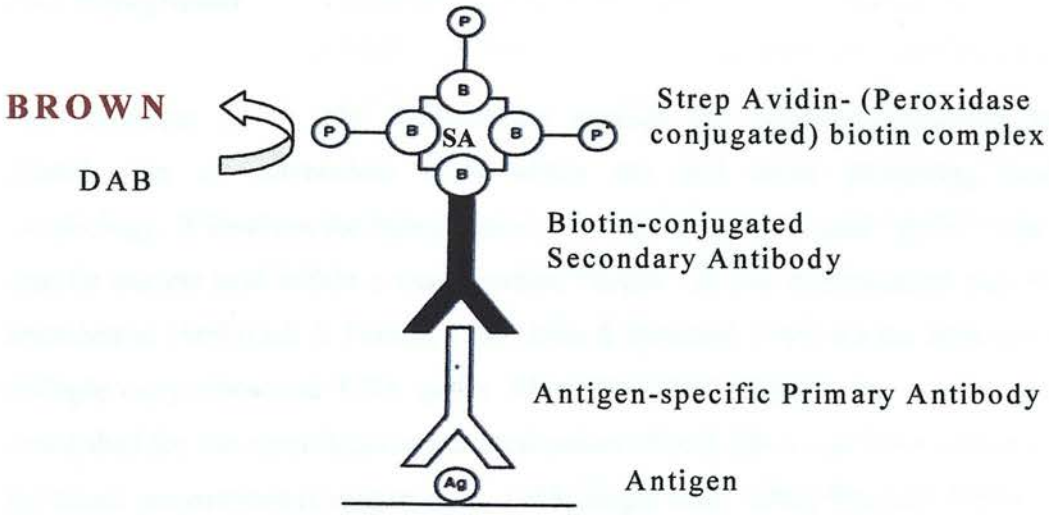
2.4.3 IHC Peroxidase protocol

The peroxidase method was used to locate ET_B receptor and β-myosin heavy chain (MHC) immunoreactivity. As described previously, wax embedded heart sections cut longitudinally to a thickness of 3μm were dewaxed in xylene, rehydrated in 100, 90 and 70% alcohol solutions and placed in water for 15 minutes. Peroxidase is present in some normal and neoplastic tissues, e.g. leucocytes and erythrocytes. Pre-incubation of sections in 0.3% hydrogen peroxide solution for 10 minutes at room temperature produces an almost complete abolition of endogenous peroxidase activity, without affecting the immunoreactivity of antigens. After incubating sections in hydrogen peroxide, sections were washed in PBS buffer for 5 minutes. Sections were then trypsinised (as described previously) at 37°C for 45 minutes, before application of a goat or rabbit blocker (plus addition of 4 drops of biotin per ml of blocker) for 30 minutes at room temperature. Unbound biotin was added to block sites of non-specific peroxidase-bound biotin occurring at later points in the procedure.

The avidin-biotin technique of indirect antibody binding is used for detection in this method (Hsu *et al.,* 1981). Avidin (molecular weight 67kdal) is a glycoprotein of egg white and is composed of four sub-units, which form a tertiary structure possessing four biotin-binding hydrophobic pockets. Biotin is a vitamin that forms the coenzyme or prosthetic group of several enzymes that transfer carboxyl groups. It is possible to conjugate proteins and other large molecules with biotin. The method to make use of avidin and biotin for immunohistochemical detection of antigens relies on the strong affinity of avidin for biotin and consists of four stages separated by washes. The first step involves addition of the primary antibody, which is unlabelled and dilute. Sections were incubated in either 1/200 mouse anti-β-MHC or 1/300 sheep anti-ET_B primary antibody made up in PBS (with addition of 4 drops of avidin per ml of antibody solution) and left overnight at 4°C. Avidin was added to block sites of non-specific binding of avidin-peroxidase bound biotin at later time points in the procedure.

The second step involves addition of a secondary antibody that is biotinylated with a large number of biotin molecules. Goat anti-mouse biotinylated IgG (1/150) for β-MHC or donkey anti-sheep biotinylated IgG (1/150) for ET_B was added to the respective sections for 30 minutes. The third step involved using a reagent that is a complex of avidin (strepavidin) with peroxidase labelled biotin. Avidin has an isoelectric point of 10, which may cause it to bind to charged sites in the tissue and lead to non-specific binding. Therefore Strepavidin (a microbial protein that contains no carbohydrate and has a more neutral isoelectric point of 7; thus less prone to attachment through charged binding sites) is used instead. After addition of the biotinylated secondary antibody, sections were incubated in the strepavidin biotin complex (Strep-ABC; Strep-ABC kit, Dako Ltd, UK) for 30 minutes (*Figure 2.8*). Strep-ABC consists of strepavidin and biotin-peroxidase which when incubated together for 30 minutes form an enormous cross-linked molecule

Figure 2.8 Diagram of Strep ABC.



In preparation of Strep-ABC, excess strepavidin in the mixture ensures that some strepavidin binding sites will be free to combine with the biotin on the secondary antibody. The strepavidin/biotin-peroxidase complex is added to the sections leading to binding of the complex to the secondary antibody and overall amplification of the peroxidase enzyme signal that can be highlighted by incubation in DAB for 20 minutes yielding a strong brown colour. Sections were finally washed in distilled water; secondary stained using haematoxylin (Harris', BDH, UK), then finally dehydrated through 70, 90 and 100% alcohol solutions and xylene before being mounted in DePeX mounting medium (Sigma, UK).

2.5 In Situ hybridisation

2.5.1 Background

The technique of *in situ* hybridisation enables the precise localisation and identification of polynucleic acids within the cell while preserving tissue morphology. It involves the hybridisation of a labelled nucleic acid "probe" with a specific nucleic acid within a tissue section "target". *In situ* hybridisation was first described in 1969 (Gall & Pardue, 1969; John & Birnstiel, 1969) for the detection of multiple copy ribosomal RNA genes. Since then the technique has become well established for the identification and localisation of both DNA and RNA within cell and tissue preparations (Coghlan *et al.*, 1985; Singer *et al.*, 1986; Warford, 1988).

In situ has two main advantages over other molecular biology techniques: precise anatomical localisation and high sensitivity. It also extends immunohistochemical results by addressing the issue of whether a positively staining cell has synthesised the detected protein or acquired it by means of uptake from the extracellular space. In studies in this thesis, detection of both ET-1 protein immunoreactivity and preproET-1 mRNA in a particular cell would provide strong evidence that the cell is the site of synthesis.

In spite of the high sensitivity and wide application of *in situ* hybridisation techniques originally developed using radioactive probes, problems were associated with safety measures required, limited shelf life, and extensive time necessary for autoradiography (around 6 weeks). However, current methods now available involving nucleic acid probes with a stable non-radioactive label overcome the major obstacles that restricted the application of *in situ*. Advantages include identification of cellular localisation of the probe by using wax embedded sections and a much shorter overall protocol duration of 4-5 days. The *in situ* method performed in this thesis involved a non-radioactive digoxygenin (DIG) labelled probe, which was highlighted using an anti-DIG alkaline phosphatase-conjugated antibody as explained below.

2.5.2 Probe synthesis & labelling

Riboprobes for detection of preproET-1, ET_A and ET_B were produced in our laboratory by Dr. Pauline McEwan. Briefly, plasmids (a gift received from Dr. Olivier Valdenaire, Hoffmann-La Roche Ltd., Basel, Switzerland) containing the cloned cDNA fragments of preproET-1, ET_A or ET_B in pCR II vector (Stratagene Ltd., CA, USA) were used to transform *E.Coli.* Ampicillin resistant cell cultures were grown up in LB media (Life Technologies Ltd., UK). The cells were then lysed and supercoiled DNA isolated and linearised using a specific restriction enzyme for the antisense or sense cDNA template required (*Table 2.4*).

Table 2.4 List of ET Probe Vectors, Promoter enzymes and Restriction enzymes.

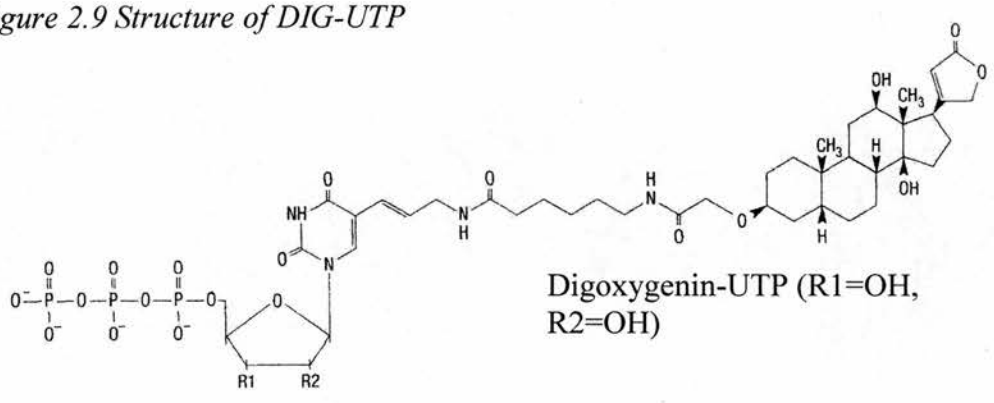
	PreproET-1	**ET_A**	**ET_B**
Vector	pCR II	pCR II	pCR II
Antisense RNA polymerase enzyme	SP6	T7	T7
Restriction Enzyme	*Eco RV*	*Kpn I*	*Kpn I*
Sense RNA polymerase enzyme	T7	SP6	SP6
Restriction Enzyme	*Kpn I*	*Eco RV*	*Eco RV*

The linearised fragments were then phenol-chloroform extracted and ethanol precipitated. Phenol denatures the proteins whilst ethanol precipitates them out to leave purified DNA. The linearised plasmid was then used as a matrix to produce labelled probe.

pCR II vector has recognition (promoter) sites for specific RNA polymerase enzymes SP6 and T7 (Promega, Madison, WI, USA). Under appropriate reaction conditions, the specific RNA polymerase enzyme (*Table 2.4*) recognises its promoter and transcribes in either the 5'→3' (antisense) or 3'→5' (sense) until it reaches the site of linearisation. The antisense strand, complementary to cellular mRNA, is an effective hybridisation probe. The sense strand, similar in length, specific activity and base composition to the cellular mRNA, provides a suitable control.

The specific RNA polymerase enzyme incorporates free ribonucleotides into an RNA transcript complementary to the cDNA insert. A DIG RNA labelling kit (SP6/T7; Boehringer Mannheim, UK) was used to perform the probe synthesis and labelling. The nonradioactive DIG, a steroid hapten, is used to label the RNA probes produced. The kit included a transcription buffer, adenosine triphosphate (ATP), cytosine triphosphate (CTP), guanine triphosphate (GTP), uracil triphosphate (UTP), DIG-UTP (DIG is linked to the C-5 position of uridine nucleotides via a spacer arm containing eleven carbon atoms (*Figure 2.9*), and SP6 or T7 polymerase.

Figure 2.9 Structure of DIG-UTP



Digoxygenin-UTP (R1=OH, R2=OH)

Linearised non-denatured template was added to a mixture of nucleotides. The mixture was then treated with DNAse (RNAse free; Boehringer Mannheim, UK) which removed residual DNA molecules leaving behind the RNA probe. Efficiency of labelling was verified on dot blot. Incorporation of DIG-UTP was one per 25 nucleotides. Synthesis of probes was carried out by Dr. Pauline McEwan. All probe-labelling procedures were carried out with the assistance of Dr. Pauline McEwan.

2.5.3 *In situ* protocol

In situ hybridisation was carried out as specified in an in-house protocol developed by Dr. Pauline McEwan (McEwan *et al.*, 1998a). Before starting the experiment, all glassware and metal racks to be used were wrapped in tin foil and baked in an oven for 3 hours at 200°C. All plastic pipette tips, ependorf tubes, and solutions including diethyl pyrocarbonate-treated water (depH_2O; see Appendix) and 10x-concentrated PBS (pH 7.6; see Appendix) were autoclaved. Paraformaldehyde solution was made up fresh (see Appendix) prior to each *in situ* experiment.

Initially, wax embedded hearts were sectioned at 3μm and floated out on a water bath set at 48°C. Sections were then placed on TESPA-coated slides and allowed to adhere overnight in an oven at 37°C. The following day, sections were dewaxed in xylene, rehydrated in 100, 90 and 70% alcohol solutions (made up with depH_2O) and before being washed twice in 1x-PBS buffer (made up with depH_2O) for 10 minute intervals.

2.5.4 Prehybridisation

The goal of the primary steps of an *in situ* hybridisation is to optimise conditions for the hybridisation reaction. Critical to the sensitivity of the *in situ* technique is the minimisation of non-specific binding. Proteins usually surround RNA target sequences in the tissue and extensive cross-linking of these proteins during fixation can mask the target nucleic acid site. Performing a number of pre-hybridisation treatments can reduce the non-specific retention of probe in tissue sections and unmask those nucleic acid sites covered by proteins. 20-30 minutes treatment with 0.2M HCl was included, which aided the extraction of proteins and partial hydrolysis of the target. Sections were also treated with the detergent Triton X-100 for 20 minutes to extract lipid membrane components still present after fixation, dehydration and embedding.

Protease treatment serves to increase target accessibility by digesting the protein that surrounds the target nucleic acid. To digest the sample, preparations were incubated with proteinase K in 20mM TE buffer (see Appendix) at 37°C. For formalin fixed heart tissue, 40μg/ml for 90 minutes was found to give optimal results.

Background staining can arise from a variety of sources, from the formation of imperfect duplexes with nonhomologous nucleic acids, from electrostatic interactions between charged groups, and from physical entrapment in the three dimensional lattice of the tissue section. Most *in situ* hybridisation protocols call for a prehybridisation step designed to decrease background binding of the probe. The prehybridisation mix includes bovine serum albumin (BSA) and 1x-Denhardts solution (Sigma, UK) to decrease non-specific binding to proteins; EDTA, salmon sperm DNA and yeast tRNA were also added to decrease non-specific nucleic acid interactions. Our prehybridisation step was carried out at the same temperature as the hybridisation step (42-48°C), for a duration of 2 hours.

2.5.5 Hybridisation

Hybridisation is the process of RNA, or DNA, re-annealing with complementary nucleic acids in solution just below their melting point. Our hybridisation mix included all the components of prehybridisation mix, as well as the DIG-labelled riboprobe and dextran sulphate. Dextran sulphate increases the rate of hybridisation by an excluded volume effect (Wahl *et al.,* 1979). In aqueous solutions, dextran sulfate is strongly hydrated. Thus, macromolecules have no access to the hydrating water, which causes an apparent increase in probe concentration and consequently higher hybridisation rates. Because of their increased stability RNA-RNA hybrids require higher hybridisation temperatures than DNA-RNA hybrids. Most hybridisation mixes contain 50% formamide, so that optimal stringencies may be obtained without the need to elevate temperatures greatly. Formamide reduces the melting temperature of DNA-DNA and DNA-RNA duplexes in a linear fashion by 0.72°C for each percent formamide. Thus, hybridisation can be performed at 30-45°C

with 50% formamide present in the hybridisation mixture. The rate of renaturation decreases in the presence of formamide.

Prolonged exposure to high temperatures causes a deterioration of cell morphology as well as loss of adherence of the section to the slide. Hybridisation was performed in the dark to prevent ionisation of formamide being enhanced by light. Labelled probe can hybridise non-specifically to sequences, which are partially, but not entirely homologous to the probe sequence. Such hybrids are less stable than perfectly matched hybrids. RNAse (DNAse free) selectively removes non-base-paired RNA from tissue sections, so that background is greatly reduced with little loss of signal. Post hybridisation washes in standard sodium citrate/formamide of various concentrations and at varying temperatures were performed, which disassociate weakly complemented hybrids. Sections were further washed in 1x DIG buffer 1 (DIG Wash & Block Buffer Kit; Boehringer Mannheim, UK) for 20 minutes before immersion in 20% sheep blocking serum (made up in 1x DIG buffer 1) for 3 hours at 24°C; this blocks non-specific binding sites of the primary antibody. Sections were then treated with an alkaline phosphatase conjugated sheep anti-DIG antibody (Boehringer Mannheim, UK) overnight at a concentration of 1/3000 (made up in 1x DIG buffer 1).

The following morning, sections were again brought through a series of 20 minute washes in 1x DIG buffer 1 and 1x DIG buffer 3 before immersion overnight in nitro-blue tetrazolium chloride/ 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP; 0.1% made up in 1x Dig buffer 3) substrate (Boeringher Mannheim, UK). PreproET-1, ET_A receptor and ET_B receptor mRNA were detected as dark blue dots within the cytoplasm of cells in heart sections. Transverse sections of aorta were used as positive controls. Negative controls were treated with RNAase prior to incubation with antisense probes. Positive controls were treated with sense probes.

PreproET-1, ET_A receptor and ET_B receptor mRNA were semi-quantified in heart sections also using the above mentioned computerised image analysis system (Image Associates, UK). Application of a frame of known area (126,015μm^2) and fixed

magnification (x20) was made to four different areas within the LV at the infarct (MI rats) or adjacent to the operation site (sham-operated control rats) and away from the infarct and in four random areas in the right ventricle (RV). Two sections from each block were cut randomly through the infarct and counted. For quantification of mRNA, data were collected from two random, consecutive heart sections from each animal for preproET-1, ET_A receptor and ET_B receptor mRNA. The intra-assay variation was <5% for each probe and therefore, data for each probe from two sections were pooled and averaged.

2.6 Data analyses

All *in situ* hybridisation data, plasma levels, organ weights, haemodynamic values and infarct parameters were presented as mean±standard error of mean (SEM). Measurements between groups were compared via one-way ANOVA, while unpaired values within groups were assessed using a Student's *t*-test. A P value < 0.05 was considered significant.

general factorial analysis

Chapter 3

Localisation of endothelin during scar formation post myocardial infarction

3.1 Introduction

There is extensive evidence in clinical studies that plasma levels of ET-1 are increased in the acute phase after myocardial infarction (MI). When compared with healthy individuals, plasma ET-1 levels were found to be significantly elevated in patients at time of admission to hospital (Miyauchi *et al.,* 1989) and remained elevated for at least 2 days after MI (Lechleitner, 1993). Another study showed that increased plasma ET-1 in patients 24 hours after the onset of acute MI correlated with higher pulmonary artery pressure and elevated pulmonary vascular resistance, while increased levels after 72 hours correlated with increased infarct size (Setsuta, 1995).

However, increased plasma ET-1 levels may be part of a systemic neurohormonal response to MI or due to a reduction in plasma ET-1 clearance. Whether increased ET-1 is synthesised *de novo* and produced locally in the infarcted heart in the early stages after MI remains to be clarified. Animal studies have demonstrated increased plasma ET-1 released from pig hearts subjected to a brief period of CAL followed by reperfusion (Tønnessen *et al.,* 1993). Furthermore, Watanabe *et al.* (1991) found a substantial increase in ET-1 in cardiac tissue up to 2 days after coronary artery ligation (CAL) and reperfusion in the rat. However, since ET-1 was determined by radioimmunoassay in plasma and tissue extracts, the histological sites of production had not been addressed in any of these studies. Furthermore, no studies have investigated the distribution of ET-1 in the infarct during the progression of scar formation after MI, without reperfusion intervention.

Within the developing scar many dynamic processes occur, including fibroblast proliferation and collagen deposition, expression of growth factors (such as TGF-β_1), new vessel formation and inflammatory cell infiltration (see section 1.3.2). *In vitro* studies have shown that ET-1 can regulate cardiac fibroblast function (Dawes *et al.,* 1996), with both ET_A and ET_B receptors evident on fibroblasts (Katwa *et al.,* 1993). Furthermore, ET-1 was found to be involved in promoting endothelial cell migration and angiogenesis *in vivo* (Goligorsky *et al.,* 1999). ET-1 also stimulates pro-

inflammatory responses in macrophages, monocytes and neutrophils in both *in vitro* (Cunningham *et al.,* 1996; Ruetten *et al.,* 1996) and *in vivo* studies (Filep *et al.,* 1995). Therefore, endogenous ET-1 may increase in the developing scar and regulate the processes of infarct healing.

The aims of this chapter were, therefore, to characterise the main processes involved in early stage scar formation, to investigate the specific localisation of endogenous ET-1 peptide in the infarct, and to examine whether distribution of endogenous ET-1 peptide changes in the infarct over the first two weeks during the development of scar formation.

3.2 Methods

All procedures were carried out as described in Chapter 2. Three study time points after CAL in the rat were investigated, namely 2 days post-CAL, 7 days post-CAL, and 14 days post-CAL; each with respective sham-operated groups. The developing scar was characterised with respect to cellular content including inflammatory cell infiltration, new vessel formation, collagen deposition, and TGF-β_1 and ET-1 immunoreactivity.

3.2.1 Coronary artery ligation rat model

MI was induced by ligation of the left anterior descending coronary artery in male Wistar rats as described in section 2.1. Upon recovery from surgery, rats were housed individually and allowed access to normal rat chow and water ad libitum for the duration of the study.

3.2.2 Plasma collection and tissue sampling

Either 2, 7 or 14 days after CAL or sham-operation, rats were anaesthetised (60mg/kg Na pentobarbital i.p.) and then exsanguinated via a needle placed in the dorsal aorta where an ~8ml blood sample was collected into a 10ml syringe pre-rinsed with heparin. The blood was aliquoted into pre-chilled test tubes containing 50µl of 10mmol/l final concentration EDTA and immediately centrifuged (2000x*g*, 4°C, 20mins). Plasma was aliquoted off each sample and stored at -70°C for future plasma ET-1 and big ET-1 analysis as described in section 2.2.2.

The heart, lungs and kidneys were excised, rinsed in ice-cooled physiological saline, then individually weighed. The heart and kidney were cut longitudinally; a section of the lung was also separated before all samples were placed in 10% neutral buffered formalin solution for 24hrs fixation prior to further processing and wax embedding.

Within the 7 day and 14 day post-CAL groups, infarct size as a percentage of the left ventricular free wall was measured from 3μM heart sections stained with van Gieson's collagen stain and the nucleus stained with Celestine Blue as described in section 2.3.1.

3.2.3 General staining and immunohistochemistry

3μm wax embedded heart sections were treated with van Gieson's collagen stain highlighting collagen, and naphthol AS-D chloroacetate esterase highlighting neutrophils as described in section 2.3.3. Immunohistochemistry was also performed on 3μm heart sections using antibodies to ET-1, endothelial cells (GSL I antibody), and TGF-β_1 using the alkaline phosphatase detection method as described in sections 2.4.2.

3.2.4 Statistical analysis

All results data were displayed as mean ± SEM. Unpaired measurements from CAL and respective sham-op groups were compared using a Student's *t*-test. A P value < 0.05 was considered significant.

3.3 Results

3.3.1 Effects of coronary artery ligation

During the study period none of the sham-operated animals died. However, in the CAL group mortality usually occurred during the 24-hour period after the ligation procedure had been performed. The average survival 24-hour post-ligation was 68%. *Table 3.1* below summarises organ weights, plasma ET-1 and big ET-1 values, and LV infarct sizes from the relevant groups.

Table 3.1 Table of organ weights, plasma ET-1 and big ET-1 levels and infarct sizes.

Group	N	Heart Weight (g/kg body weight)	Lung Weight (g/kg body weight)	Plasma ET-1 (pg/ml)	Plasma big ET-1 (pg/ml)	Infarct Size (% of LV free wall)
2 day CAL	5	4.61±0.24	5.23±0.38	2.29±0.21	16.47±1.3	
2 day sham	4	4.04±0.12	4.50±0.27	1.78±0.17	17.32±4.1	
7 day CAL	6	3.74±0.09	4.43±0.36	2.10±0.15	22.98±3.0	41.1±10.2
7 day sham	3	3.41±0.19	4.49±0.32	1.81±0.04	18.02±2.5	
14 day CAL	3	3.37±0.18	4.05±0.45	1.76±0.34	25.26±2.1	33.5±5.2
14 day sham	4	3.35±0.16	4.09±0.53	2.26±0.36	17.00±2.6	

CAL did not significantly alter mean heart weight (HW), lung weight (LW), plasma ET-1 or plasma big ET-1 values versus the respective sham-operated groups at any of the 3 time points, though there was a tendency for increased plasma big ET-1 values in CAL groups at the 2 later time points. Heart weights were expected to be higher in the CAL groups. However, a smaller number in some of the groups may have contributed to the non-significant values observed. Infarct size measured as a percentage of the LV free wall was moderate in both 7 and 14-day CAL groups, with no significant difference between the 2 groups.

3.3.2 Histological and immunohistochemical characterisation of normal myocardium.

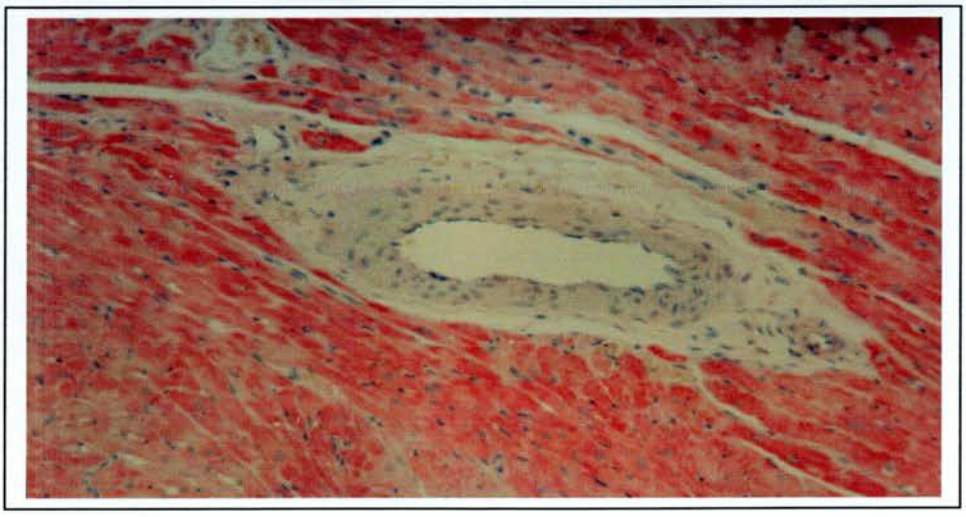
The staining distribution of van Gieson's collagen stain in normal myocardium is shown in *Figure 3.1 (a)*. Cardiomyocytes, myoendothelial cells and vascular smooth muscle stained yellow, whereas the collagenous adventitial layer of the vessel and interstitial tissue collagen levels stained pink. Interstitial collagen levels were low in normal myocardium.

ET-1 immunoreactivity was found to be diffusely distributed in the cytoplasm of cardiomyocytes, myoendothelial cells and some vascular endothelial cells throughout normal myocardium of the rat heart (*Figure 3.1 (b)*). Vascular smooth muscle cells in the myocardium did not show ET-1 immunoreactivity. An immunohistochemical protocol was performed using an identical IgG primary antibody that had no recognition site for ET-1, with no staining evident in any areas of the myocardium (*Figure 3.1 (c)*). Furthermore, ET-1 immunoreactivity in the myocardium of hearts from unoperated rats was similar to that displayed in myocardium of sham-op rats (*Figure 3.1 (d)*).

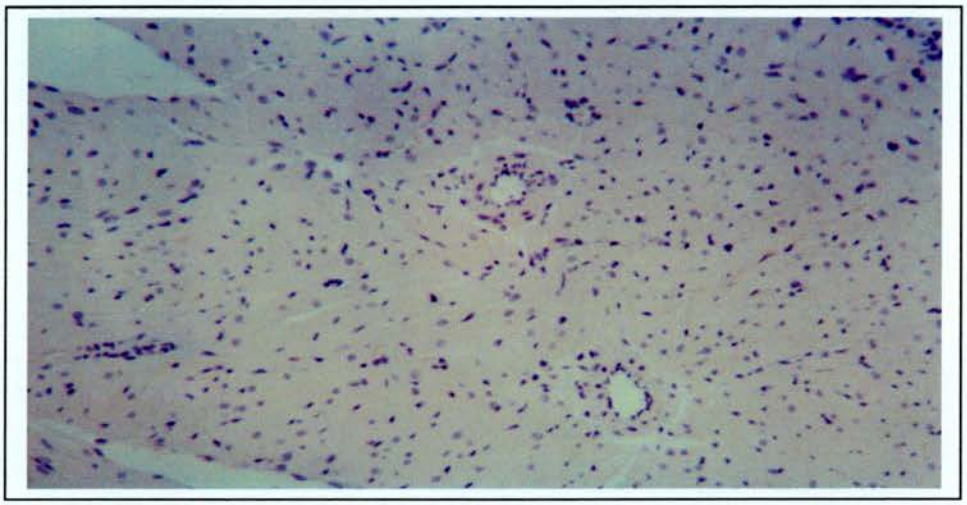
Figure 3.1 Images of normal myocardium staining from sham-operated and unoperated rat hearts. van Gieson's stain highlights collagen in pink and normal myocardium in yellow, with nuclei counterstained dark blue with haematoxylin. ET-1 immunoreactivity is shown in red, with nuclei counterstained with haematoxylin. (a) van Gieson's collagen staining (x200 magnification); (b) ET-1 immunohistochemical staining (x400 magnification); and (c) an ET-1 negative control using a non-specific primary IgG antibody (x200 magnification), all from sham-operated rat hearts; (d) ET-1 immunohistochemical staining (x200 magnification) from a freshly killed unoperated rat heart.

(a)

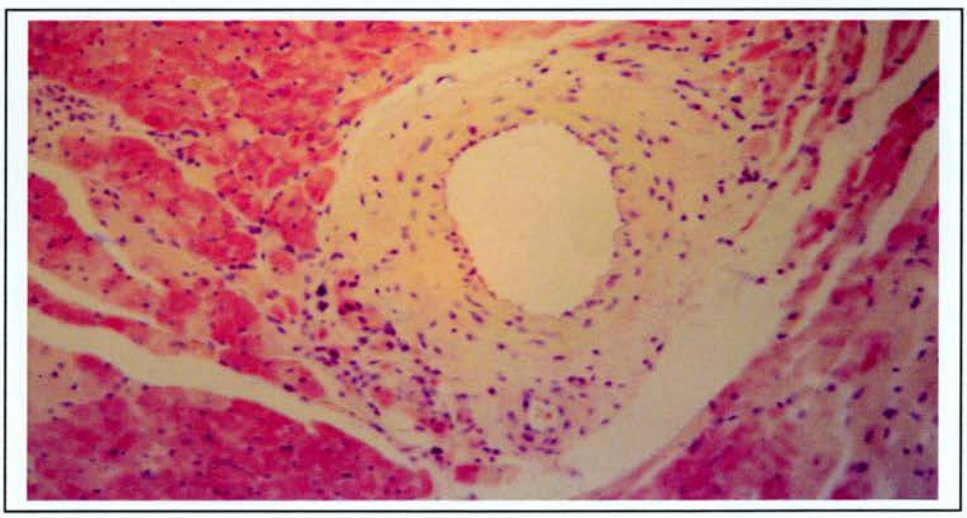
(b)



(c)



(d)



3.3.3 Histological and immunohistochemical characterisation of the developing scar

3.3.3.1 2 days post-MI

Figure 3.2 (a) displays an area of the LV affected by the ligation. At 2 days, within the infarct, there were areas of cellular necrosis identified by cells that have a disrupted cell wall and cell morphology (*Figure 3.2 (b)*). There were still some viable myocytes present (*Figure 3.2 (c)*), though these tended to be situated close to, or surrounding, vessels that might still be receiving some blood supply. There was also a marked increase in number of cells, which were most probably inflammatory cells and proliferating fibroblasts, throughout the infarcted myocardium *Figure 3.2 (d)*, though fibroblasts were not synthesising collagen at this time.

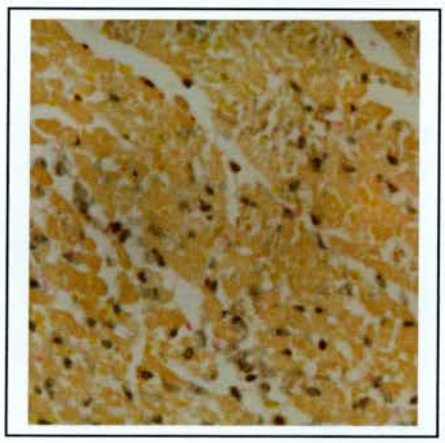
Within the infarct at 2 days post-CAL, areas of cell death and tissue necrosis showed faint endothelial cell staining using GSL I (*Figure 3.3 (a)*), indicating a disrupted capillary supply to the affected tissue. This was in contrast to the defined morphology of myoendothelial cells that formed a capillary network throughout normal myocardium (*Figure 3.3 (b)*). Some larger vessels that served the myocardium of the infarcted area also showed GSL I immunoreactivity in their endothelial cell layer (not shown). However, there was little evidence of angiogenesis in any areas of the infarct at this time point.

As mentioned in Section 2.4.2, GSL I was also found to be an excellent marker for inflammatory cells as well as endothelial cells. Within the infarct, many inflammatory cells were evident, bordering and infiltrating central areas of necrosis. The larger stained cells (*Figure 3.4 (a), arrow*), displaying a similar morphology to macrophages, were distinguishable from endothelial cells by their larger size, more rounded cell shape, and a defined circular nucleus. Furthermore, these cells were not evident in normal myocardium.

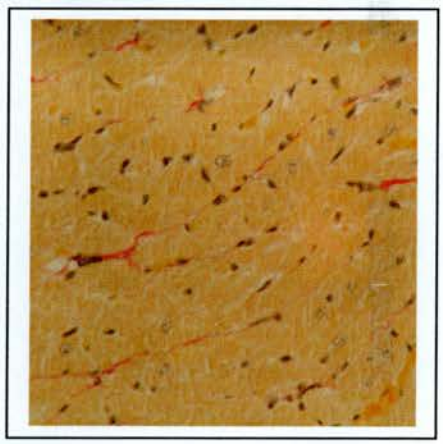
Figure 3.2 Images in the infarcted LV from hearts in the 2-day CAL group stained with van Gieson's stain. (a) overview of the infarcted area (x50 magnification); and (b, ★) necrotic tissue ; (c, †) viable myocardium; (d, ✦) increased cellular content; within the infarcted myocardium (all x400 magnification).

(a)

(b)



(c)



(d)

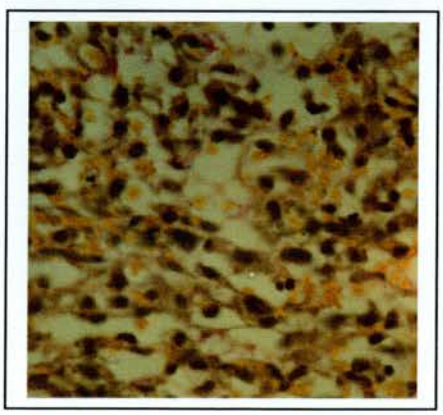
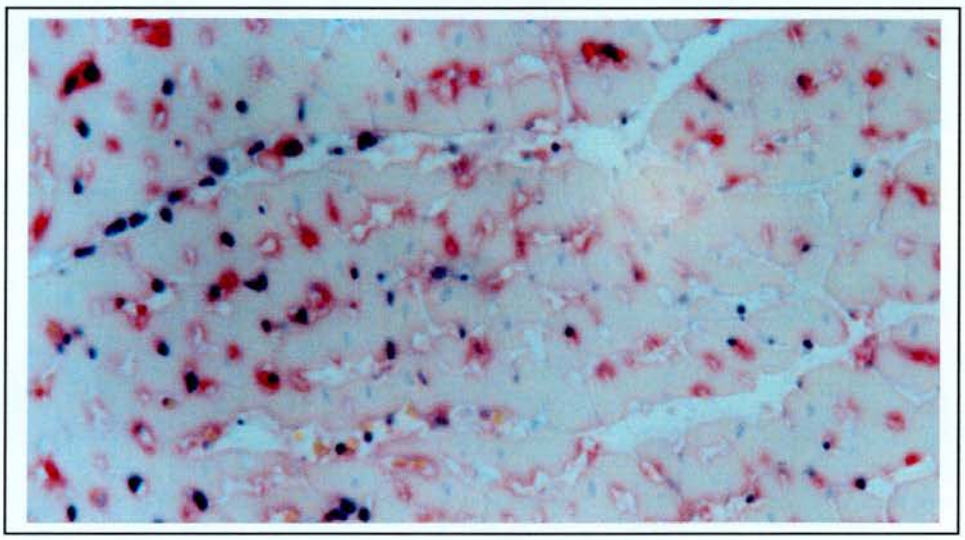


Figure 3.3 Images showing GSL I staining of endothelial cells in red and nuclei stained purple with haematoxylin. (a) loss of defined endothelial capillary staining within necrotic tissue of the infarct at 2 days post-CAL (x400 magnification); (b) GSL I highlighting the defined morphology of endothelial cells that form a capillary network throughout normal myocardium (x1000 magnification).

(a)



(b)

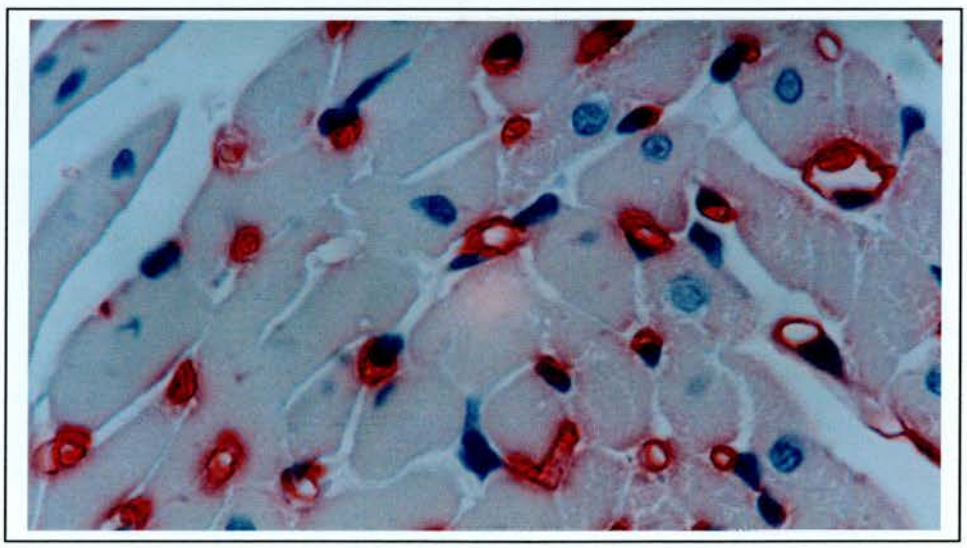
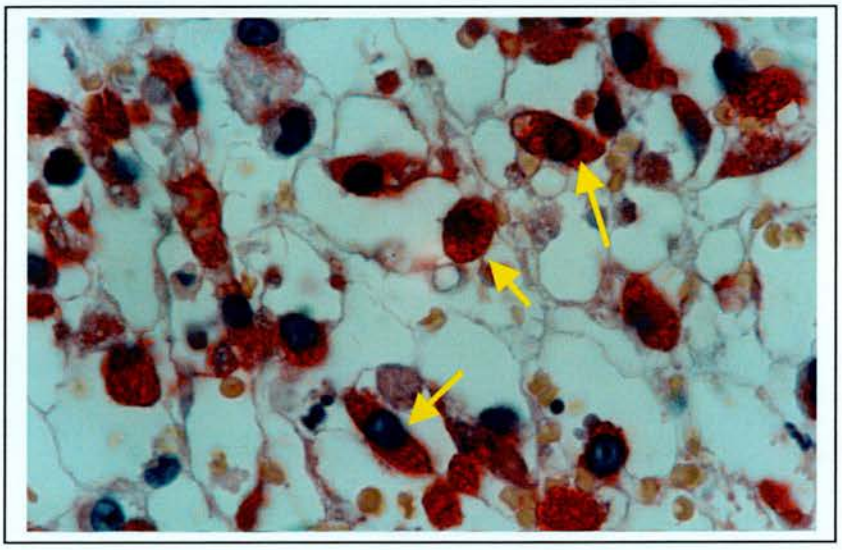
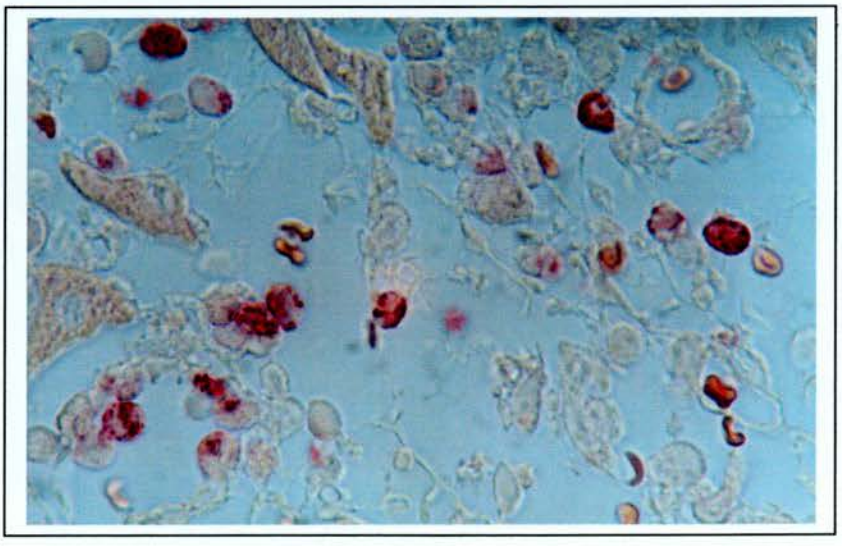


Figure 3.4 Inflammatory cells within the infarct at 2 days post-CAL. (a) Macrophages (arrows) were stained red using GSL I with nuclei staining purple with haematoxylin (x1000 magnification); (b) Neutrophils stained purple with naphthol AS-D chloroacetate esterase stain, nuclei were not stained due to similarity in colour with the cytoplasmic stain (x1000 magnification).

(a)



(b)



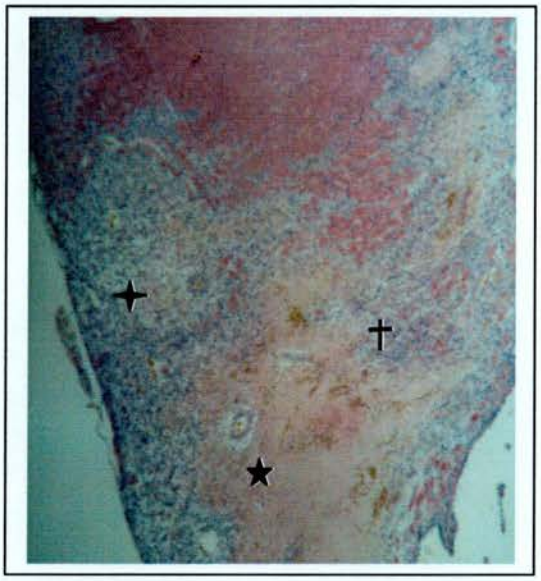
Neutrophils, whose cytoplasm stained purple with naphthol AS-D chloroacetate esterase (*Figure 3.4 (b)*), were evident in large numbers in the infarct and correlated directly with areas of necrosis at the border and in central regions of the scar. Neutrophils were also observed in some of the smaller vessels within necrotic areas indicating that these vessels, in central areas of the scar, still received blood flow. There was no evidence of neutrophils in the non-infarcted LV, RV or septum of CAL rats or in any areas of sham-op myocardium (not shown).

ET-1 immunoreactivity in the non-infarcted LV (*Figure 3.5 (a)*), RV and septum was similar to that observed in sham-op myocardium. However, within the infarct, cells undergoing necrosis showed faint ET-1 immunoreactivity (*Figure 3.5 (b)*). ET-1 staining was detected in macrophages (*Figure 3.5 (c), filled arrowhead*) and neutrophils (*Figure 3.5 (d)*) within the infarct, though fibroblasts tended not to display ET-1 immunoreactivity at this time point. (*Figure 3.5 (c), open arrowhead*).

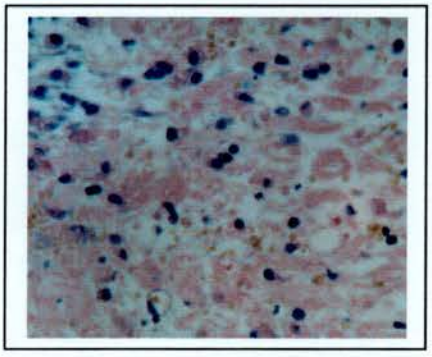
Myocardium affected by the ligation showed no TGF-β_1 immunoreactivity at this time point. However, viable cardiomyocytes within the infarct and those at the border and above the infarct in the LV displayed increased TGF-β_1 staining (*Figure 3.6 (a)*) compared to that observed in the RV and septum of CAL rats or in any areas of sham-op myocardium (*Figure 3.6 (c)*). TGF-β_1 staining in vascular smooth muscle cells was not evident. Interestingly, TGF-β_1 immunoreactivity also correlated with distribution of ET-1 staining in cardiomyocytes, both within the infarct and at the border (*Figure 3.6 (b)*). TGF-β_1 immunoreactivity in myocardium from sham-op rats (*Figure 3.6 (c)*, and from rats that underwent no operative procedures (not shown), demonstrated faint cytoplasmic staining thoughout the myocardium located to cardiomyocytes and myoendothelial cells.

Figure 3.5 Images representative of hearts from the 2-day post-CAL group. (a) overview area of infarcted LV and non-infarcted border showing ET-1 immunoreactivity (x50 magnification); (b, ★) area of necrosis showing faint ET-1 staining (x400 magnification); (c, ✦) area of increased cell content showing ET-1 staining in macrophages (filled arrowheads) but not in fibroblasts (open arrowheads, x400 magnification); and (d, †) neutrophils showing ET-1 immunoreactivity (x1000 magnification).

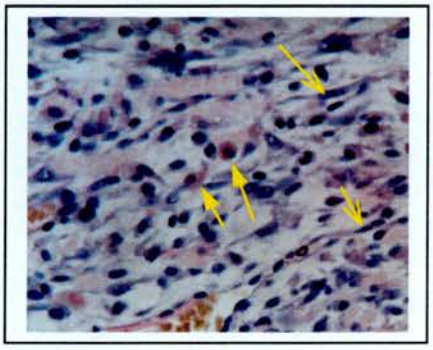
(a)



(b)



(c)



(d)

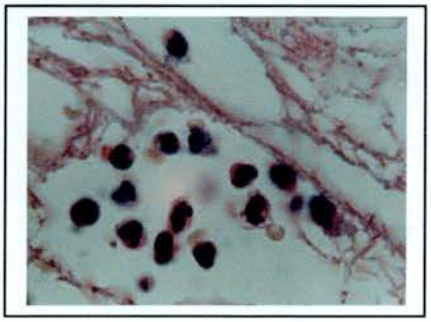
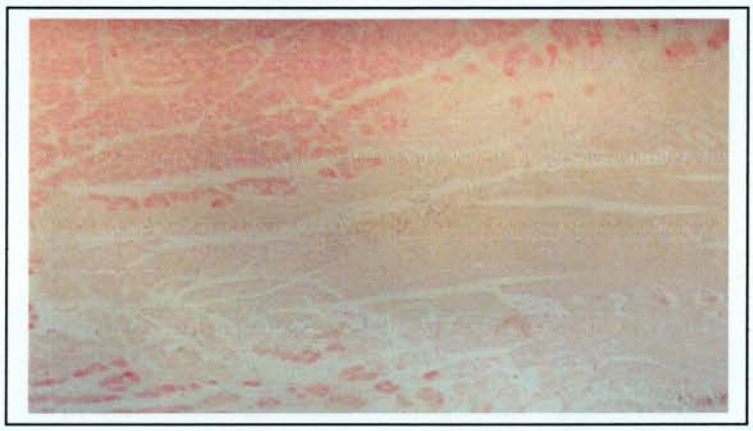
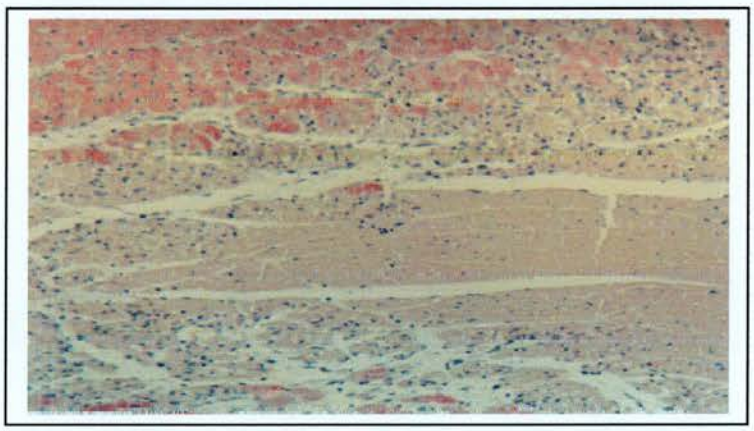


Figure 3.6 Images representative of TGF-β_1 staining (pink) in the LV of hearts from the 2-day CAL group. (a) increased TGF-β_1 staining in cardiomyocytes in the non-infarcted LV, but a loss of staining in tissue affected by the MI (x200 magnification); (b) similar distribution of ET-1 immunoreactivity in a parallel section (x200 magnification); and (c) TGF-β_1 staining in myocardium of a sham-op rat heart (x400 magnification).

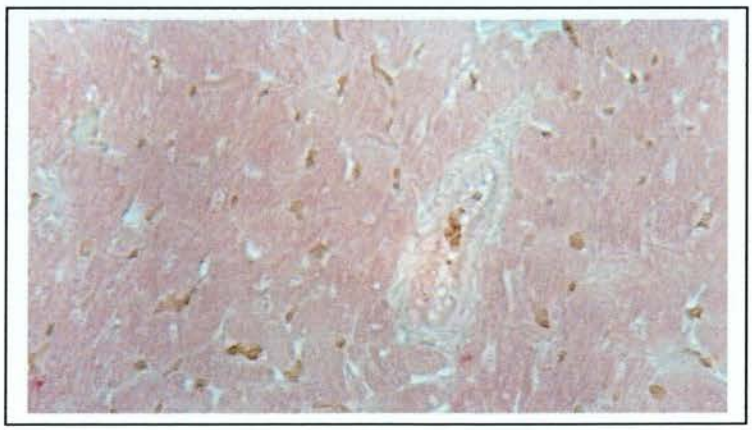
(a)



(b)



(c)



3.3.1.2 7 days post-MI

At 7 days post-CAL, necrotic tissue is being cleared from within the infarct and proliferating fibroblasts are now beginning to synthesise collagen as shown by pink staining in *Figure 3.7 (a)*. At this time point, synthesised collagen is an immature type that has not formed cross-linking fibrils.

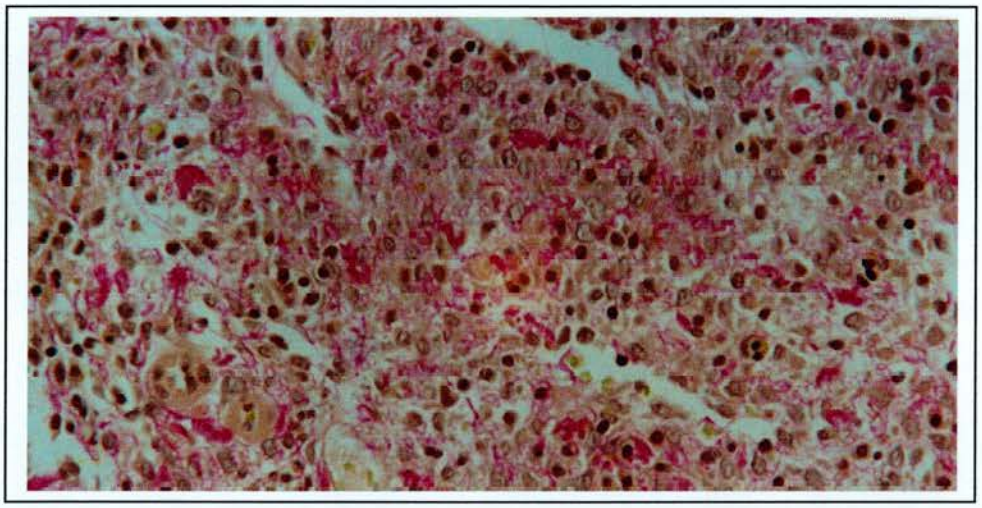
GSL I immunoreactivity was observed in the scar and could be located in the endothelia of numerous newly forming vessels (*Figure 3.7 (b)*). New vessels appeared mostly in areas undergoing repair and scar formation. These were most evident on the outer edge of the infarct at this time point, though a few could also be found in some central areas of necrotic tissue.

Inflammatory cells, also stained by GSL I, were highlighted in areas where necrosis, degradation and cell clearance were still present, namely in central areas of the scar (*Figure 3.7 (c)*). Macrophages *(arrows)*, identified as large cells with a distinct circular nucleus, were the predominant inflammatory cell in the scar at this time, though monocytes (smaller rounded cells with a central circular nuclei), and some neutrophils (bilobed nuclei) were also evident (not shown). GSL I also highlighted non-specific staining of red blood cells, though these could be easily distinguished from inflammatory cells by their smaller size and lack of a nucleus.

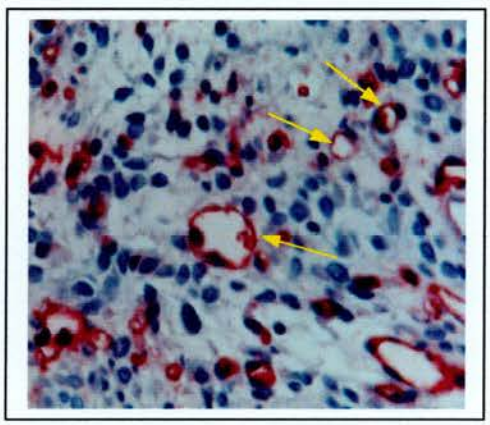
Increased distribution of ET-1 immunoreactivity was detected throughout the developing scar at 7 days post-CAL (*Figure 3.8 (a)*). ET-1 could be localised in remaining cardiomyocytes (not shown), but also fibroblasts (*Figure 3.8 (b), open arrowhead*), numerous infiltrating inflammatory cells (*Figure 3.8 (b), closed arrowhead*), and in endothelial cells of newly forming vessels (*Figure 3.8 (b), dotted arrow line*). ET-1 staining in the non-infarcted LV was similar to that observed in the RV and septum of CAL rats and in all areas of sham-op rat myocardium (data not shown).

Figure 3.7. Sections representative of areas within the infarct at 7 days post-CAL. (a) collagen synthesis (pink) highlighted with van Gieson's stain (x200 magnification); (b) angiogenesis (arrows, x400 magnification) and (c) macrophages (arrows, x400 magnification) highlighted within necrotic tissue using GSL I (red).

(a)



(b)



(c)

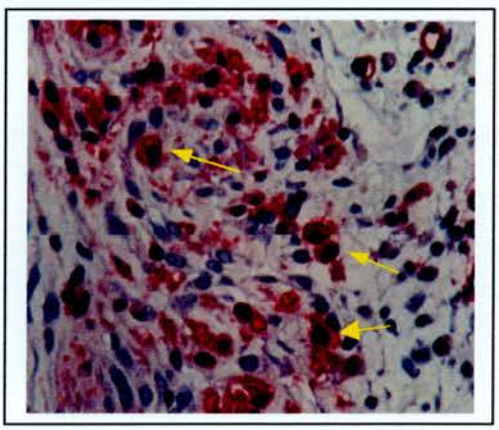
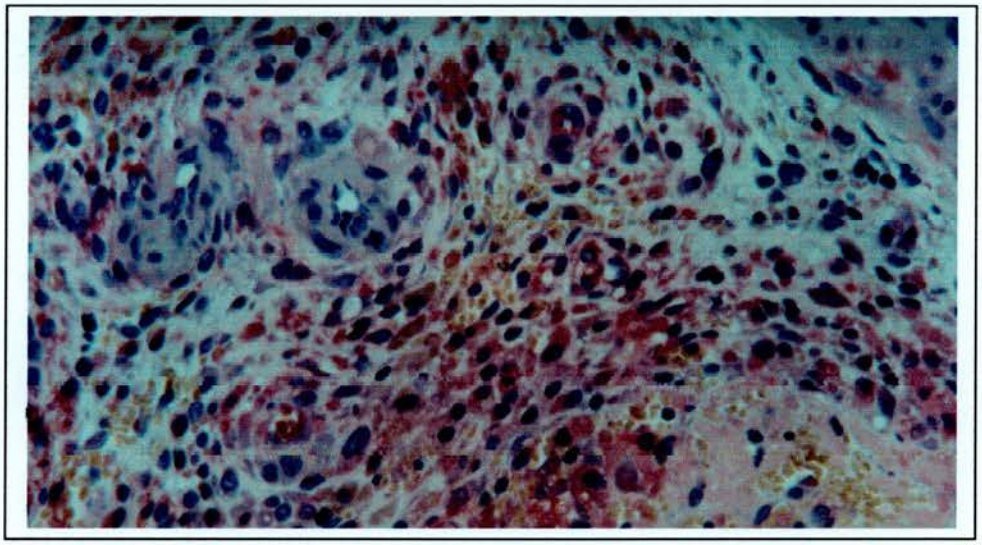
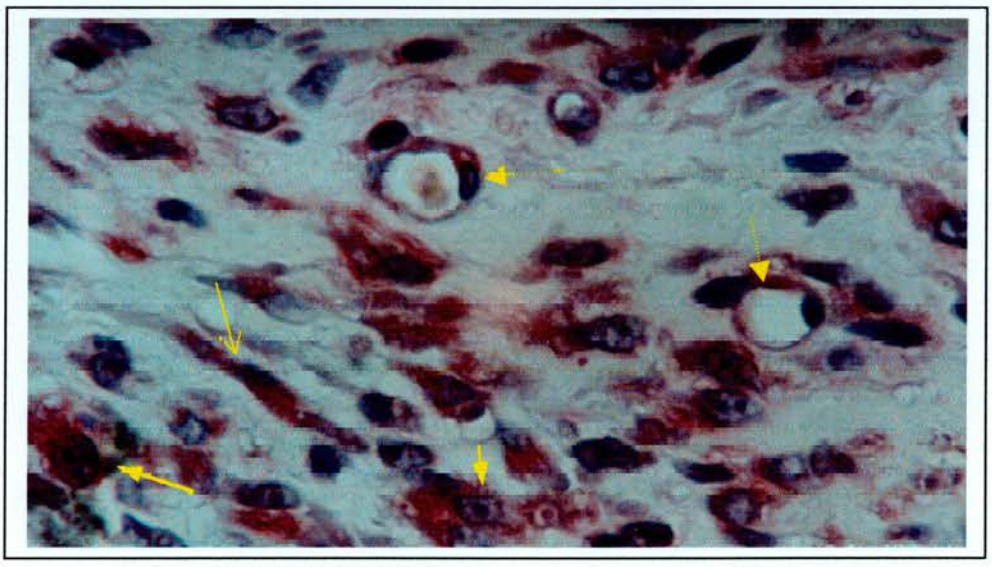


Figure 3.8 Sections showing ET-1 immunoreactivity in the infarct, representative of that found within scars in the 7-day post-CAL group. (a) Increased distribution of ET-1 immunoreactivity throughout infarct compared to that observed at 2 days post-CAL (x200 magnification); (b) ET-1 staining could be located in fibroblasts (open arrowheads), inflammatory cells (closed arrowheads) and in the endothelia of newly forming vessels (dotted arrow line, x1000 magnification).

(a)



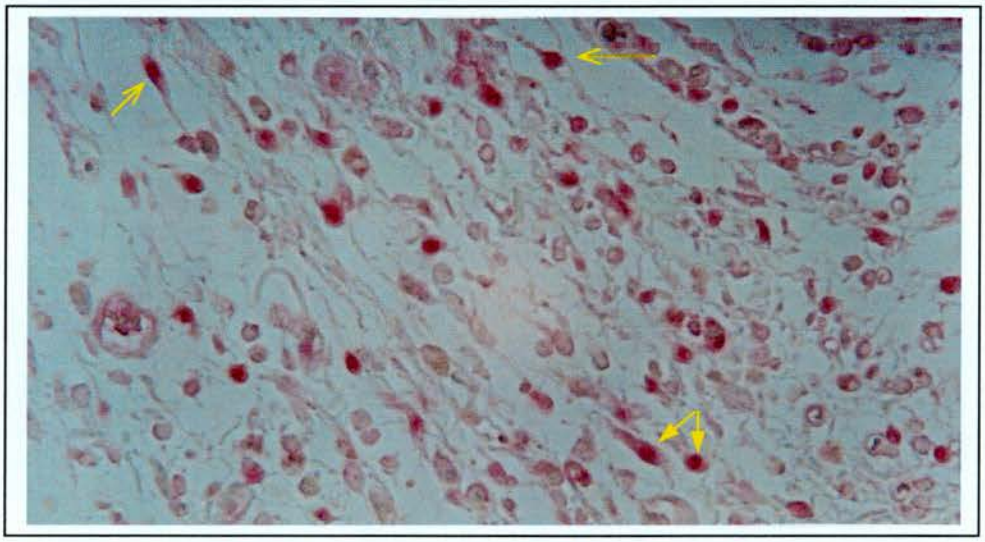
(b)



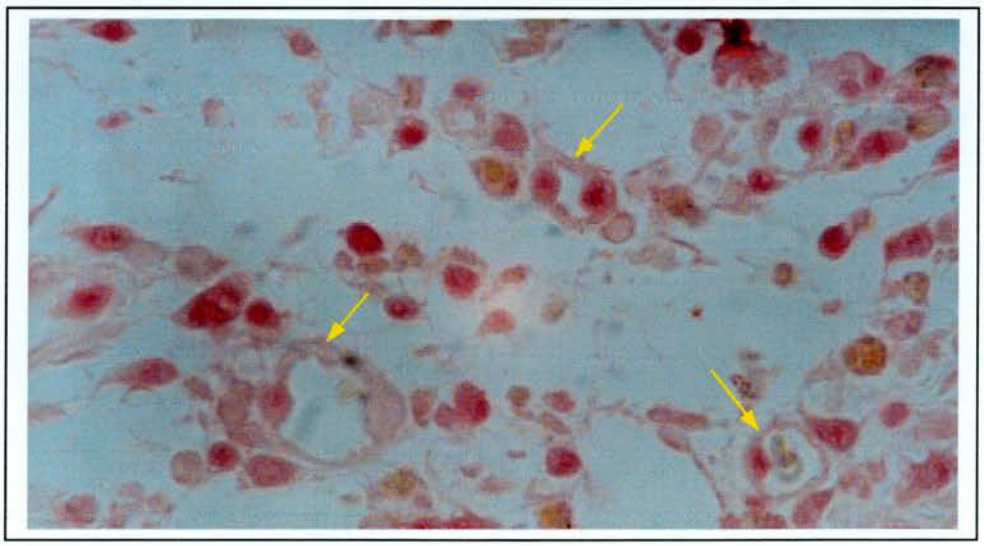
TGF-β_1 immunoreactivity was increased in the infarct compared to 2 days and could be located in specific cells throughout the developing scar (*Figure 3.9 (a)*). These included fibroblasts (*Figure 3.9 (b), open arrowhead*), inflammatory cells (*Figure 3.9 (b), closed arrowhead*) and in the endothelia of newly forming vessels (*Figure 3.9 (b), dotted arrow line*). Furthermore, TGF-β_1 immunoreactivity was also evident in those cells that displayed ET-1 immunoreactivity. However, TGF-β_1 staining in these cells was observed around the nuclei, with little staining of the cytoplasm. This was in contrast to the staining found in cardiomyocytes, which was throughout the cytoplasm. In the non-infarcted LV, TGF-β_1 immunoreactivity was increased throughout the cytoplasm of cardiomyocytes at the border of the infarct, compared to that found in the RV and septum (data not shown). Staining in the RV and septum of CAL rats was similar to that found in sham-op rat myocardium (data not shown).

Figure 3.9 Sections showing TGF-β_1 immunoreactivity (red) within the infarct of hearts representative of the 7-day CAL group. (a) TGF-β_1 staining could be located around the nucleus of fibroblasts (open arrowheads) and in macrophages (closed arrowheads, x400 magnification) and (b) in the endothelia of newly forming vessels (arrows, x1000 magnification).

(a)



(b)



3.3.1.3 14 days post-MI

In those hearts in which a large MI had occurred after CAL, a thin scar was formed after 14 days, which consisted predominantly of collagen and a few remaining areas of viable myocytes (*Figure 3.10 (a)*). Deposited collagen was in the form of mature cross-linking fibrils (*Figure 3.10 (b)*) distinct from that observed at 7 days, and constitutes the main tensile component of the infarct wall.

GSL I staining was still evident in the endothelia of small vessels established throughout the scar. These vessels could be found both on the outer edge and in central areas of the infarct (data not shown).

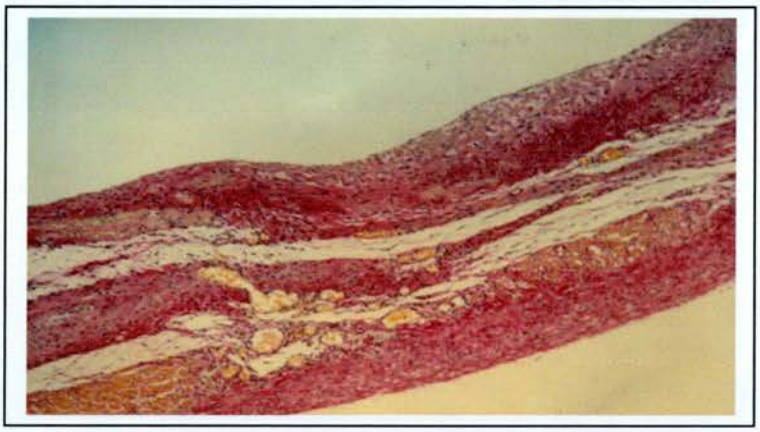
Inflammatory cells were evident only in central areas of the scar where remaining degradation and clearance of necrotic tissue still occurred (*Figure 3.10 (c) arrows*). Again, the predominant inflammatory cells within the scar at this time were macrophages.

ET-1 immunoreactivity was evident only in remaining viable cardiomyocytes (*Figure 3.10 (d)*) and a few inflammatory cells in the thinned scar (not shown). However, in those hearts where ligation resulted in a smaller MI, the formation of a mature scar was not complete, and ET-1 immunoreactivity could still be located in many inflammatory cells (*Figure 3.10 (e), filled arrowheads*) and fibroblasts (*Figure 3.10 (e), open arrowheads*) within the infarct. ET-1 staining in the non-infarcted LV was similar to that observed in the RV and septum of CAL rats and also to that in sham-op rat myocardium (not shown).

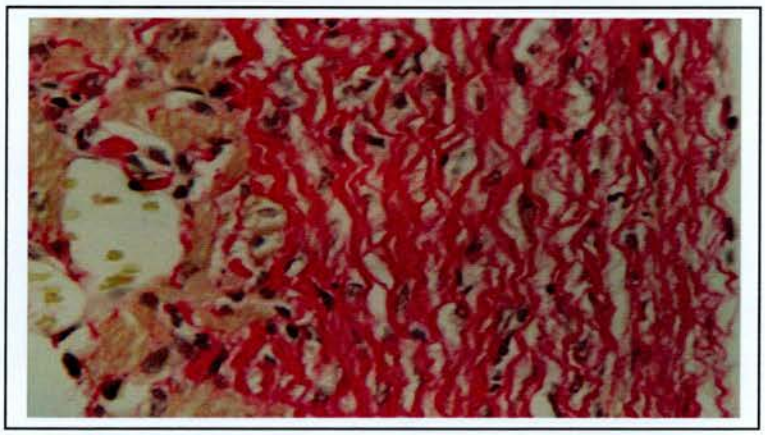
In developing scars at 14 days, TGF-β_1 staining could be located in inflammatory cells (*Figure 3.10 (f), closed arrowhead*), fibroblasts (*Figure 3.10 (f), open arrowhead*) and in the endothelia of newly forming vessels (*Figure 3.10 (f), dotted arrow line*). However, in those hearts that had a thinned scar, TGF-β_1 staining was less evident, being located in remaining inflammatory cells and a few fibroblasts (not shown).

Figure 3.10 Images (all from within the infarct) of heart sections representative of the 14-day CAL group. (a) van Gieson's stain (x100 magnification) and (b) x400 magnification; (c) inflammatory cell immunoreactivity (arrowheads showing macrophages, x200 magnification); (d) ET-1 immunoreactivity (x100 magnification) and (e) in fibroblasts (open arrowheads), macrophages (closed arrowheads, x400 magnification); (f) TGF-β_1 immunoreactivity in fibroblasts (open arrowhead), macrophages (closed arrowhead) and endothelia (dotted arrow line; x400 magnification).

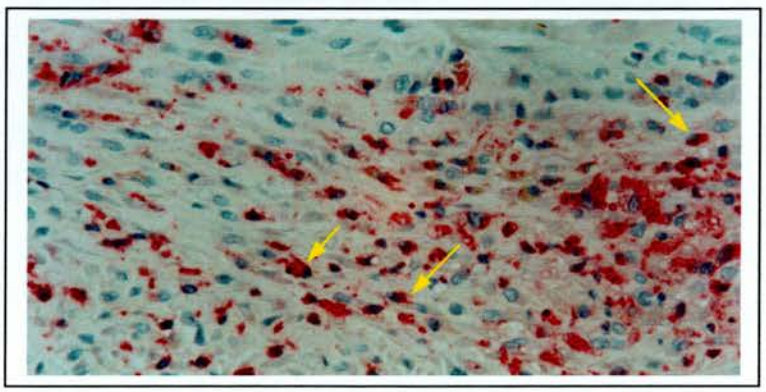
(a)



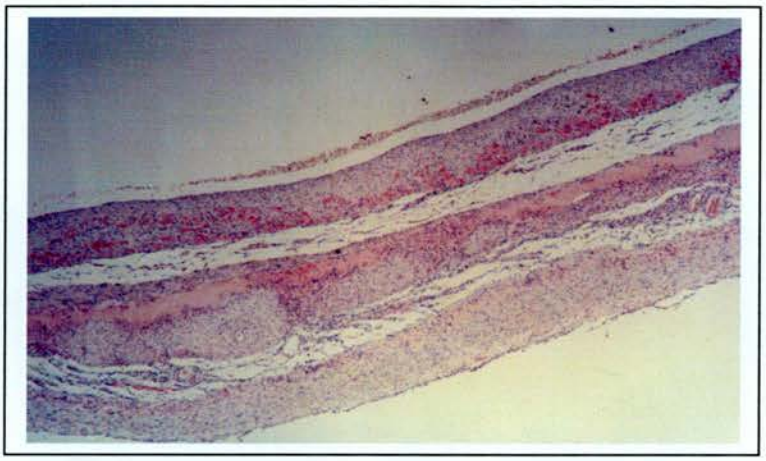
(b)



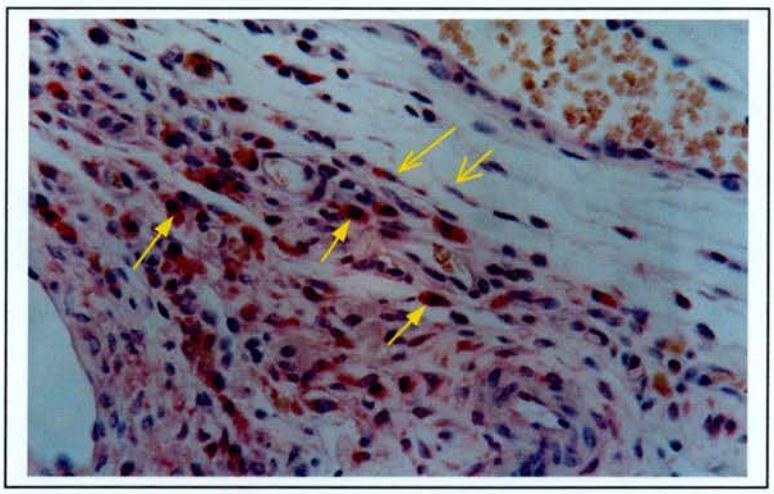
(c)



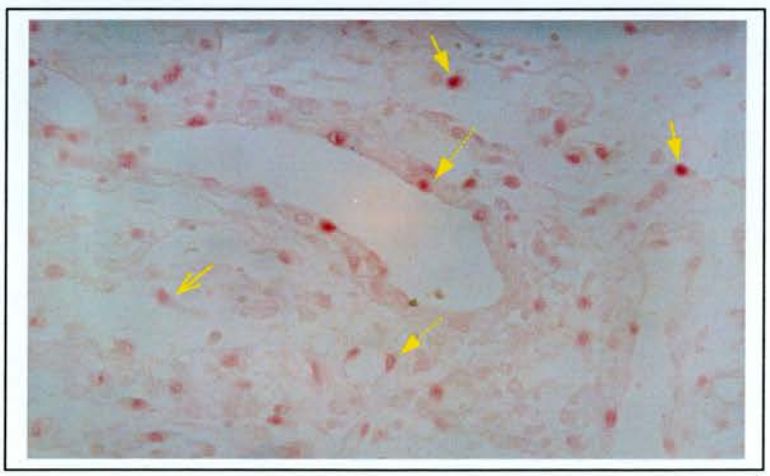
(d)



(e)



(f)



The following table summarises the changes in detection of specific peptides and processes involved in scar formation over the first 14 days post-MI.

Table 3.2 Detection levels of specific peptides and processes involved in scar formation over the first 14 days post-MI. Key: Low (+), medium (++), or high (+++) levels of detection.

	Infarct		
	2 days	7 days	14 days
Neutrophils	+++	+	
Macrophages	+++	++	+
Collagen		++	+++
Angiogenesis		+++	++
ET-1	+	+++	++
TGF-β_1	+	+++	++

3.4 Discussion

Within normal myocardium, ET-1 immunoreactivity could be located to cardiomyocytes, myoendothelial cells and some vascular endothelial cells indicating the presence of mature ET-1 peptide is present in these cells under normal physiological conditions. Sections of heart from sham-op rats showed similar distribution of ET-1 immunoreactivity to that found in the hearts from male Wistar rats that underwent no operative procedures, indicating that surgical processes did not account for the distribution of immunoreactivity observed. At all 3 time points post-CAL, ET-1 staining in the non-infarcted LV was of similar intensity to that in the RV and septum and also similar to that found throughout the myocardium of sham-op rat hearts.

Distribution of ET-1 staining observed in sham-op hearts was similar to that shown in other studies using sham-op rat hearts compared against CAL groups (Øie *et al.*, 1997; Tønnessen *et al.*, 1998). Furthermore, evidence of ET-1 staining in cardiomyocytes has also been reported in hearts from sham-op rats in other CAL studies (Sakai *et al.*, 1996a; 1996b; Kobayashi *et al.*, 1999), and in cardiomyocytes from endocardial biopsy specimens of transplanted human hearts (Giaid *et al.*, 1995). However, a few studies have shown ET-1 immunoreactivity in endocardial endothelial cells, but not in cardiomyocytes, in the human right ventricle (Plumpton *et al.*, 1996), fetal human hearts (Giaid *et al.*, 1991; Wharton *et al.*, 1991) and adult rat hearts (Giaid *et al.*, 1991). Interestingly, a sample of ET-1 antibody from the Davenport laboratory was used on heart sections from the studies in this thesis, and highlighted ET-1 immunoreactivity throughout the myocardium, similar to that observed with our own antibody (a comparison of immunoreactivity between the two antibodies is shown later, in Chapter 5).

Within the infarcted LV, an increased number of ET-1 immunoreactive staining cells was found at 7 days compared to 2 days post-CAL, before remaining constant or lowering at 14 days post-CAL depending on whether formation of a mature scar was complete. Peak ET-1 staining occurred within the infarct at 7 days post-CAL and could be localised to areas of granulation and remaining viable myocytes.

Granulation tissue staining included fibroblasts, inflammatory cells, and endothelial cells that were involved in formation of new capillaries. These findings agree with recent studies that also showed increased ET-1 immunoreactivity in the infarcted LV at 7 days post-CAL in the rat (Øie *et al.,* 1997; Tønnessen *et al.,* 1998). Furthermore, Øie *et al.* (1997) demonstrated increased preproET-1 mRNA levels in the infarct during the first 6 weeks after induction of MI, with levels peaking at 7 days.

Interestingly, ET-1 immunoreactivity was not observed in fibroblasts at 2 days post-CAL, but later at 7 days when these cells were synthesising collagen. Recent *in vivo* studies investigating scar formation 7 days after MI in the rat have located ET-1 immunoreactivity in fibroblasts (Øie *et al.,* 1997; Tønnessen *et al.,* 1998). There are some potential explanations why ET-1 may be raised in fibroblasts during scar formation at this time. It is possible that the increased cellular ET-1 immunoreactivity observed, coinciding with a time when collagen is being synthesised, may indicate a role for ET-1 in regulating this process. Cardiac fibroblasts have been shown to express both ET_A and ET_B receptors, with ET_B receptors being predominant (Katwa *et al.,* 1993). *In vitro* studies have demonstrated that fibroblasts can synthesise ET-1 when stimulated by other cardiac vasoactive factors (King *et al.,* 1996; Gray *et al.,* 1998). Furthermore, administration of ET-1 to fibroblasts *in vitro* led to an 8-fold increase in type I collagen mRNA after 6 hours, which could be completely inhibited by the protein kinase C (PKC) inhibitor staurosporine indicating activation of an ET receptor-mediated pathway (Guarda *et al.,* 1993). The requirement of PKC in the ET-1 signalling pathway suggests that Gq is an upstream mediator of this response, and cardiac fibroblasts express Gq in large quantities (Hansen *et al.,* 1995). In a rat model of MI, Gq was significantly upregulated in border and scar tissue, these areas being abundant in fibroblast content (Weber & Brilla, 1991). The fact that ET-1 is produced by, and can act on, fibroblasts suggests both a paracrine and autocrine role for this peptide in collagen synthesis during scar formation.

Although ET-1 has been shown to induce fibroblast proliferation *in vitro* (Piacentini *et al.,* 2000), ET-1 immunoreactivity was faint in proliferating fibroblasts at 2 days

suggesting that it may not have a central regulatory role in this process *in vivo*. Furthermore, another study using human cardiac fibroblasts *in vitro* showed that ET-1, via the ET_A receptor, stimulated collagen synthesis without having a mitogenic effect on the fibroblasts themselves (Hafizi *et al.*, 1998). However, fibroblasts also express receptors for Ang II with stimulation of AT_1 receptors leading to collagen synthesis and stimulation of AT_2 receptors involved in mitogenesis (Smits *et al.*, 1992; Unger *et al.*, 1998). ACE and renin expression are also increased in the infarct after acute MI (Passier *et al.*, 1995; Passier *et al.*, 1996). However, the precise interactions between Ang II or ET-1 and fibroblast proliferation and collagen synthesis during scar formation remain to be clarified.

ET-1 has also been reported to be involved in the conversion of fibroblasts to myofibroblasts, which are important contractile components in the developing scar. These cells have intermediate features between those of fibroblasts and smooth muscle cells, and may temporarily express α-smooth muscle actin (Darby *et al.*, 1990). Contraction of the newly formed connective tissue promotes healing by facilitating closure of the wound margins (Villaschi & Nicosia, 1994). However, ET-1 may only be upregulated in fibroblasts as a consequence of increased progressive mechanical stress or increased humoral stimulation via Ang II and/or catecholamines, with no crucial role in the processes of infarct healing.

At 2 days post-CAL, increased TGF-β_1 immunoreactivity was observed in viable cardiomyocytes bordering the infarct and in remaining viable cardiomyocytes within the infarct, with a loss of staining in cells affected by infarction. This finding was similar to that reported in other studies where TGF-β_1 mRNA expression and/or protein were increased in remaining viable cardiomyocytes bordering and within the infarct, with a loss of staining in cells affected by the infarct at 1 (Casscells *et al.*, 1990) and 2 (Thompson *et al.*, 1988) days after MI in the rat, suggesting a role for TGF-β in the infarct healing process. Furthermore, TGF-β_1 mRNA levels were also significantly increased in the adjacent non-infarcted myocardium, but not in remote non-infarcted myocardium, of rats 2 days after MI (Hanatoni, *et al.*, 1998). Increased TGF-β_1 immunoreactivity in the non-infarcted LV adjacent to the infarct, observed

during scar development in the present study, indicates that TGF-β_1 may be activated in response to an increase in progressive wall stress and/or workload in the remaining myocardium. TGF-β_1 has been shown to promote cardiomyocyte hypertrophy *in vitro* via inducing the re-expression of fetal genes (such as β-MHC), characteristic to the expression observed in hypertrophy induced by pressure overload (Parker *et al.*, 1990a). However, β-MHC expression was not investigated here.

Increased TGF-β_1 immunoreactivity found within the infarct at 7 days is also in agreement with a recent study that found peak TGF-β_1 mRNA expression in the infarcts of rats 7 days post-MI (Sun *et al.*, 2000). Other studies have also demonstrated peak levels of both TGF-β in rat wound chambers after 7 days (Cromack *et al.*, 1987) and of connective tissue growth factor (a peptide selectively induced by TGF-β) in the infarct tissue of 7 day post-MI rats (Ohnishi *et al.*, 1998). However, none of the above studies revealed the cellular location of TGF-βs within the scar at this time point, whereas the present study localised TGF-β_1 peptide to endothelial cells, fibroblasts and inflammatory cells within the granulation tissue. There is abundant information regarding the upregulation of TGF-β_1 during cardiac fibrosis (Sun *et al.*, 1998; Tomita *et al.*, 1998). However, its regulatory role in fibrous tissue formation and the precise mechanisms by which TGF-β_1 is itself upregulated remains unclear. Under hypoxic conditions, cultured human cardiac fibroblasts treated with TGF-β_1 synthesise collagen I (Agocha *et al.*, 1997). TGF-β_1 also increases synthesis of α-smooth muscle actin in myofibroblasts, which as mentioned earlier, are important in contraction of the scar during healing (Desmouliere *et al.*, 1993). Furthermore, TGF-β receptors are upregulated at the site of MI and colocalise in areas with fibroblasts and macrophages at 1 week (Sun & Ramires, 1996). In the present study, the appearance of TGF-β_1 immunoreactivity and synthesis of collagen within the infarct were both observed at 7 days but not 2 days post-CAL.

During inflammation, there is evidence that TGF-β_1 becomes upregulated as a mechanism to suppress further inflammatory cell responses (Kulkarni *et al.*, 1993).

TGF-β can be synthesised by macrophages (Wahl *et al.,* 1990), by B and T lymphocytes as well as by smooth muscle cells (Kirschenlohr *et al.,* 1993). TGF-βs have, therefore, been suggested to be factors that shift the healing process from inflammation, towards fibrosis (Nicoletti & Michel, 1999). The observation of TGF-β_1 immunoreactivity in inflammatory cells at 7 days post-CAL in the present study coincides with the time of downgrading of the inflammatory response in rat hearts post-MI (Cleutjens *et al.,* 1999). TGF-β_1 has also been found to stimulate angiogenesis in endothelial cells *in vivo* during wound healing (Fajardo *et al.,* 1996) and evidence of TGF-β_1 staining in these cells at 7 days post-CAL in the present study may suggest a role in microvessel formation in the infarct at this time.

The predominant mechanism by which TGF-β_1 becomes upregulated in inflammatory cells, fibroblasts and endothelial cells in the developing scar at this specific time remains to be clarified. There is evidence that Ang II can stimulate synthesis of TGF-β_1 in fibroblasts (Lee *et al.,* 1995). Ang II was also found to stimulate cardiac myocyte hypertrophy via paracrine release of TGF-β_1 from fibroblasts (Gray *et al.,* 1998). As previously mentioned Ang II levels are increased in the infarct after MI, though it is not known if ET-1 has a role in regulating TGF-β_1 expression in these cells. Interactions between TGF-β_1 and ET-1 have been reported in other studies outwith the heart. TGF-β_1 was found to stimulate the expression of ET-1 mRNA by vascular endothelial cells *in vitro* (Kurihara *et al.,* 1989), and stimulate the release of ET-1 from stellate cells in the rat liver *in vitro* (Gabriel *et al.,* 1999). Alternatively, cyclosporin was found to amplify ET-1 production, which in turn promoted the synthesis and activation of TGF-β_1 in the rat kidney (Hutchinson, 1998). Interestingly, in the present study, those specific cells within the infarct at 7 days post-CAL that showed increased TGF-β_1 immunoreactivity also showed elevated ET-1 immunoreactivity. However, whether ET-1 is an important regulator of TGF-β_1 peptide synthesis is unclear. Equally, TGF-β_1 may have a regulatory role on synthesis of ET-1 instead.

At 2 days post-CAL, ET-1 immunoreactivity could be localised to the cytoplasm of invading macrophages and neutrophils within the necrotic tissue. ET-1 immunoreactivity was also evident in the cytoplasm of varied inflammatory cells within the infarct at 7 days post-CAL including macrophages, and also some monocytes and lymphocytes. *In vitro* culture studies and *in vivo* studies in pathological diseases have shown that inflammatory cells can synthesise and be stimulated by ET-1 (Ehrenreich *et al.,* 1990; Chanez *et al.,* 1996; Cunningham *et al.,* 1997). However, the precise role of upregulated ET-1 in these cells is unclear. ET-1 has been shown to have both pro-inflammatory (McMillen *et al.,* 1995) and anti-inflammatory activities (King *et al.,* 1997). However, whether upregulated ET-1 acts to reduce or maintain the inflammatory response in the developing scar post-MI in the rat remains to be clarified.

Neovascularisation was prominent in the infarct at 7 days post-MI and has been suggested to be an efficient mechanism for restoring coronary flow to the affected myocardium (Nelissen-Vrancken *et al.,* 1996). Evidence of a role for ET-1 in angiogenesis is, however, controversial. ET-1 has been reported to induce angiogenesis via an ET_A receptor mediated pathway in endothelial cells in the rat cornea (Bek & McMillen, 1999). Another *in vitro* study found a dose-dependent stimulatory proliferative and migratory effect on endothelial cells isolated from bovine adrenal capillaries, which was mediated through the ET_B receptor only (Ziche *et al.,* 1995). Also, Goligorsky *et al* (1999) demonstrated that the mitogenic and angiogenic effects of ET-1 are mediated via the ET_B receptor and that endothelial nitric oxide synthase (NOS) is requisite for this action. However, Stewart *et al.* (1996) showed decreased levels of ET-1 and increased NOS activity during angiogenesis. In this study, ET-1 immunoreactivity was observed in the endothelia of newly forming vessels, which appeared at the boundary and within the granulation tissue of the infarct at 7 days post-CAL. The localisation of ET-1 in these vessels suggests that it may regulate angiogenesis at this time point. However, whether ET-1 stimulates proliferation, migration and the formation of tubules from these endothelial cells, or conversely, is involved in reducing further unwarranted vessel formation in the developing scar is not known.

In summary, the localisation of ET-1 in fibroblasts producing collagen, endothelial cells forming microvessels, and inflammatory cells clearing necrotic tissue suggests ET-1 may have an active role in the developing infarct. However, whether upregulated ET-1 is beneficial or detrimental to scar formation during this early time period after MI remains to be established. Furthermore, that both TGF-β_1 and ET-1 immunoreactivity is upregulated in these cells at a specific time point during scar development suggests an interaction between these peptides, though whether ET-1 is an important regulator of TGF-β_1 synthesis, or vice versa, is not known.

Chapter 4

Investigation of the effect of non-selective endothelin receptor antagonism on scar formation post myocardial infarction

4.1 Introduction

In the previous chapter, ET-1 immunoreactivity was shown to increase within the infarct over the first 2 weeks during scar formation with the number of ET-1-stained cells highest at 7 days. At this time, ET-1 staining could be localised in fibroblasts synthesising collagen, inflammatory cells involved in clearance of necrotic tissue, and endothelial cells that were involved in formation of new capillaries. However, whether increased ET-1 has beneficial or detrimental effects on these active processes of scar formation is not known.

A number of studies have used ET receptor antagonists to investigate the role of ET-1 within the heart during the early stage post-myocardial infarction (MI). However, a large variation in study conditions occurs, including the experimental animal model used (coronary artery ligation (CAL)/reperfusion *in vivo* model, Langendorff *in vitro* model); the type of ET receptor antagonism (ET_A, $ET_{A/B}$); time of, and duration of ET receptor antagonist administration; and responses measured (arrhythmias, haemodynamic changes, area of myocardial damage/infarct size).

No study to date has investigated directly the effects of endogenous ET-1 on the active processes involved in *in vivo* scar formation post-MI. Many antagonist studies however have used a CAL/reperfusion model. This model, depending on the timing of reperfusion after coronary occlusion, produces animal models with varying degrees of myocardial damage. This is different to the model used in the present study, in which irreversible occlusion leads to major myocardial damage in the affected area with blood flow re-entering the infarct, in the initial stages, most probably via collateral flow (Maxwell *et al.*, 1987). In the CAL/reperfusion model, beneficial, neutral and detrimental effects of ET receptor antagonism have been reported in animal models early post-MI, depending on class of antagonist used and time of administration (Grover *et al.*, 1993; McMurdo *et al.*, 1993; Kojima *et al.*, 1995; Vitola *et al.*, 1996).

The published literature, therefore, suggests that ET-1 may have an active role in events occurring in the heart early post-MI including haemodynamic changes and determination of infarct size. However, it is not known whether ET-1 has a direct role on the active processes of scar formation. Therefore, the aim of this chapter was to investigate the role of increased ET-1 on the processes of scar formation following CAL in the rat using a non-selective ET receptor antagonist A-182086 (Abbott Laboratories, Illinois, USA) administered immediately upon recovery from CAL, and continued daily for 2 weeks during scar formation.

4.2 Methods

All procedures were carried out as described in Chapter 2. Three study time points after coronary artery ligation (CAL) or sham-operated (sham-op) in the rat were investigated, namely 2, 7 and 14 days, each with respective antagonist fed and control diet groups (see *Table 2.2*). Scar parameters measured included infarct size as a percentage of the LV free wall, infarct thickness, and myocardium to collagen ratio within the infarct. The developing scar was also characterised with respect to cellular content including inflammatory cell infiltration, new vessel formation, collagen deposition, TGF-β_1 and ET-1 immunoreactivity, and TUNEL positive staining.

4.2.1 Coronary artery ligation rat model

MI was induced by ligation of the left anterior descending coronary artery in male Wistar rats (n=45) as described in section 2.1. After surgery and before recovery from anaesthesia, a tail-tip blood sample was taken for plasma analysis of LDH levels as described in section 2.2.1. A value 3 times greater than that found in plasma from a corresponding sham-op rat indicated a successful ligation. Upon recovery from anaesthesia, rats were started on the antagonist protocol where individual rats were randomly fed either food containing the non-selective ET receptor antagonist A-182086 (30mg/kg rat/day) or food without the antagonist, as described in section 2.3.3, for the duration of the study.

A-182086 exhibits good oral bioavailability and substantial plasma half-life ($t_{½}$=8.1 hr at a single oral dose of 10mg/kg) in the rat. A-182086 has also been shown to inhibit S6c-induced depressor responses in anaesthetised rats at ≥3mg/kg and to dose dependently inhibit pressor responses to big ET-1 at ≥10mg/kg (data from Abbott Laboratories, USA). Previous pilot studies in this thesis (section 2.3.2) showed that in rats fed A-182086 (30mg/kg/day) upon recovery from CAL or sham-op surgery and thereafter daily in their food over 2 days; both ET_A and ET_B-mediated responses to a bolus intravenous injection of ET-1 in anaesthetised rats were nearly completely inhibited.

4.2.2 Haemodynamic measurements, plasma collection and tissue sampling

Either 2, 7 or 14 days after CAL or sham-op, rats were anaesthetised (60mg/kg Na pentobarbital) and the right carotid artery located, dissected free of extraneous tissue and cannulated with a polyethylene fluid filled catheter attached to a pressure transducer, for measurement of MAP and LVEDP as described in section 2.2. Rats were then exsanguinated via a needle placed in the dorsal aorta from which an ~8ml blood sample was collected into a 10ml syringe pre-rinsed with heparin. The blood was aliquoted into pre-chilled test tubes containing 50µl of 10mmol/l EDTA and immediately centrifuged (2000x*g*, 4°C, 20mins). Plasma was aliquoted off each sample and stored at -70°C for future plasma ET-1 and big ET-1 analysis as described in section 2.2.2.

The heart and lungs were excised, rinsed in ice-cooled physiological saline, then individually weighed. The heart was then bisected longitudinally from apex to base so that each half consisted of both right and left ventricles; a section of the lung was also separated before all samples were placed in 10% neutral buffered formalin solution for 24hrs fixation prior to further processing and wax embedding. Within the 7 day and 14 day CAL and CAL + A-182806 groups, infarct size as a percentage of the left ventricular free wall was measured from 3µm heart sections stained with van Gieson's collagen stain and the nucleus stained with Celestine Blue as described in section 2.3.1.

4.2.3 General Staining and immunohistochemistry

3µm wax embedded heart sections were treated with either van Gieson's collagen stain, a neutrophil stain naphthol AS-D chloroacetate esterase (Sigma, UK), or a TUNEL positive cell marker (Dead-End Colometric Apoptosis Detection System, Promega, UK) as described in section 2.2.3.4. TUNEL-positive cells were counted using a computerised image analysis system; Zeiss Kontron 300, Image Associates, UK. Immunohistochemistry was also performed on 3µm heart sections using

antibodies to ET-1, endothelial cells and inflammatory cells (GSL I antibody), and TGF-β_1 using the alkaline phosphatase method as described in sections 2.4.2. The apoptosis assay was performed with the assistance of Mr. David O'Regan.

4.2.4 *In situ* hybridisation

In situ hybridisation was performed (as described in section 2.5) to investigate 1) whether expression of preproET-1, ET_A and ET_B receptor mRNA altered within the heart during scar formation, and 2) if expression was affected by administration of A-182086. However, problems arose during the protocol including non-specific staining and poor quality of highlighted signal. Many attempts were made to overcome these problems including re-ordering new blocking solution, primary antibody and hybridisation buffer. Also, concentrations of antibody, blocker and substrate were changed along with incubation periods for each step. Unfortunately, these problems could not be overcome in the timeframe set aside for carrying out these studies, and the experiment was therefore stopped.

4.2.5 Statistical analysis

All data were expressed as mean ± standard error of mean. Multiple group measurements were compared via one-way ANOVA, while unpaired values within groups were assessed using a Student's *t*-test. A P value ≤ 0.05 was considered significant.

4.3 Results

4.3.1 Effects of coronary artery ligation and antagonist treatment.

For rats undergoing CAL, a plasma LDH value 3x that found in plasma from a sham rat operated on the same day indicated a successful ligation. During the study period none of the sham-operated animals died. However, in the CAL group mortality usually occurred during the 24-hour period after the ligation procedure had been performed. The average survival 24-hour post-ligation was 69%. Out of the 69% that survived, 9% of CAL-operated rats had no infarct. Of those rats that died, there was no significant difference in number of deaths between antagonist treated or untreated rats (data not shown). Within the antagonist treated groups, 2 CAL rats did not eat within the first 24 hours after surgery, and were therefore excluded from the study.

4.3.1.1 Organ weights and haemodynamic parameters

At the 2 and 7-day time points, average heart weight (g/kg body weight) in the CAL group was significantly higher compared to that in the sham-op group, though administration of A-182086 in the CAL group did not significantly alter the value at either time point (see *Table 4.1* for all data). At the 14-day time point, average heart weight in the CAL group was significantly higher compared to that in the sham-op group. Addition of the non-selective ET receptor antagonist A-182086 to the CAL group significantly reduced heart weight compared to that in the CAL group alone. However the value in the antagonist treated group was still significantly higher than that found in the sham-op group (*Table 4.1*), with or without antagonist.

At 2 days and 14 days post-CAL, mean arterial blood pressure (MAP) was significantly lower compared to their respective sham-op groups. At 7 days post-CAL, MAP also tended to be lower compared to the respective sham-op group. Addition of A-182086 to rats in the 7 and 14-day CAL groups had no effect on MAP. However, the significance observed in the 2 day CAL group versus the respective sham-op group was lost in the presence of antagonist (*Table 4.1*).

Left ventricular end-diastolic pressure (LVEDP) was significantly increased in the 14-day CAL group compared to the sham-op group. Addition of A-182086 in the CAL group did not significantly alter LVEDP. There were no significant changes in LVEDP values in either the 2-day or the 7-day groups (*Table 4.1*). Measurement of LVEDP was not accomplished for all rats due to difficulties in directing the cannula into the left ventricle.

Table 4.1 Effect of CAL and antagonist treatment on organ weights and haemodynamic parameters for the 12 groups studied.

Group	n	Heart Weight (g/kg body weight)	Lung Weight (g/kg body weight)	MAP (mmHg)	LVEDP (mmHg)
2 day CAL	8	4.52±0.20*	6.28±0.72	85.02±5.40¥	8.73±0.77 (n=3)
2 day CAL + A-182086	6	4.31±0.19*	4.90±0.19	93.81±10.83	2.46 (n=2)
2 day sham	8	3.60±0.08	4.98±0.09	109.11±6.73	3.97±0.80
2 day sham + A-182086	6	3.46±0.11	5.11±0.14	100.51±5.93	5.65±0.54
7 day CAL	8	3.89±0.07*¶	5.63±0.48	90.71±6.11φ	7.56±1.61 (n=4)
7 day CAL + A-182086	6	3.88±0.23	5.57±0.65	106.41±5.80	8.57±1.93 (n=4)
7 day sham	8	3.36±0.04	5.28±0.11	104.4±9.63	6.24±0.65 (n=3)
7 day sham + A-182086	7	3.62±0.08*	5.11±0.08	112.49±7.92	3.62±0.92 (n=3)
14 day CAL	8	4.15±0.18*†#	6.89±1.02	88.59±7.78¶ϕ	10.17±3.04ϕ
14 day CAL +A-182086	7	3.60±0.12*	5.31±0.23	85.80±3.71¥	8.23±2.71
14 day sham	8	3.23±0.05	4.78±0.07	110.84±6.89	2.28±0.67 (n=4)
14 day sham+A-182086	7	3.35±0.07	4.70±0.06	111.75±4.22	3.52±1.79 (n=3)

Key: Using one-way ANOVA, heart weights were significantly different between the 2-day ($P<0.01$), 7-day ($P<0.02$), and 14-day ($P<0.001$) groups. MAP was significantly different between the 14-day groups ($P<0.01$). Within groups, unpaired comparisons were significantly different as follows: *$P<0.005$ versus respective sham group; ¥$P<0.05$ versus respective sham group; ϕ$P=0.05$ versus respective sham group; †$P<0.005$ versus respective sham + A-182086 group; ¶$P<0.05$ versus respective sham + A-182086 group; φ$P=0.05$ versus respective sham + A-182086 group; #$P<0.05$ versus respective CAL + A-182086 group. Group sizes = 6, 7, or 8 unless otherwise stated.

4.3.1.3 Plasma ET-1 / big ET-1 values

There were no significant changes in plasma ET-1 or big ET-1 values between CAL and sham-op groups at any of the 3 time points studied (*Table 4.2*). Addition of A-182086 to CAL and sham-op groups significantly increased plasma ET-1 levels compared to respective untreated groups at all 3 time points studied, but had no effect on plasma big ET-1 values. Due to difficulties encountered in measuring plasma big ET-1, it was not possible to obtain values for all rats in the group.

Table 4.2 Effect of CAL and antagonist treatment on plasma ET-1 and big ET-1 levels for the 12 groups studied.

Group	n	Plasma ET-1 (pg/ml)	n	Plasma big ET-1 (pg/ml)
2 day CAL	7	5.46±1.25	4	9.49±6.73
2 day CAL + A-182086	5	16.87±4.36†	1	3.14
2 day sham	6	3.89±1.13	3	1.85±0.47
2 day sham + A-182086	7	17.36±2.92*	4	6.83±2.60
7 day CAL	8	5.81±0.42	6	9.41±1.37
7 day CAL + A-182086	4	18.27±3.75*	3	8.19±1.24
7 day sham	6	5.66±0.88	2	6.97±2.05
7 day sham + A-182086	4	14.67±2.06*	3	10.25±2.32
14 day CAL	5	4.40±0.43	3	6.62±1.14
14 day CAL + A-182086	6	14.81±1.61*	-	-
14 day sham	5	4.54±0.36	5	4.18±0.53
14 day sham + A-182086	6	12.57±2.72*	4	2.81±0.67

Key: Using one-way ANOVA, plasma ET-1 values were significantly different between the 2-day ($P<0.001$), 7-day ($P<0.0001$) and 14-day ($P<0.001$) groups. Within groups, unpaired values were significantly different as follows: *$P<0.05$ versus respective untreated group; †$P=0.05$ versus respective untreated group.

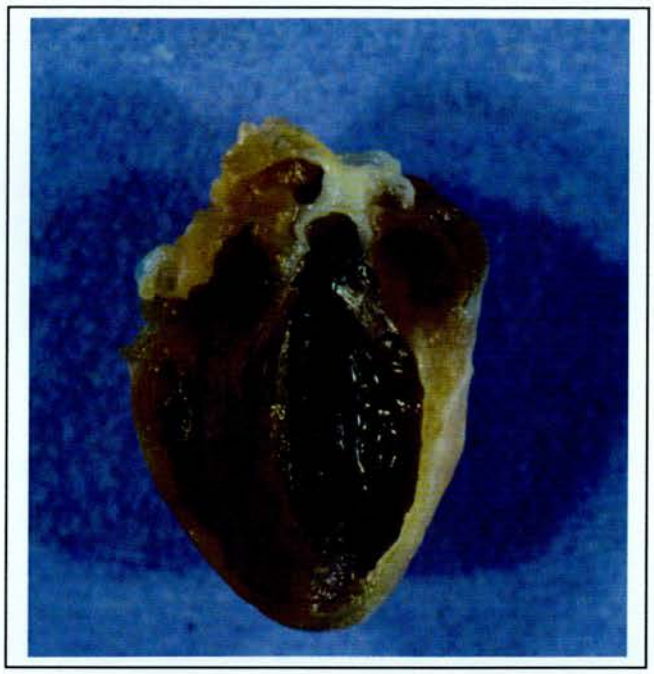
4.3.1.3 Gross morphology of hearts from CAL and CAL + A-182086 group rats

Hearts from rats in the untreated group generally had a thinned, defined, infarct 14 days post-CAL surgery, similar to that observed in hearts from the 14-day CAL group in chapter 3. *Figure 4.1 (a)* shows a cross-section of heart, cut apex to base, from a rat in the 14-day CAL group, which displays a thinned, mature collagenous scar in the area of the LV affected by the ligation. Above the scar is the area of non-infarcted LV, which may undergo hypertrophy and remodelling during the progression to heart failure. Again, as previously noted in chapter 3, a variation in the rate of development of a mature scar was observed in some of the hearts at both 7 and 14 days post-CAL, presumably due to the slight variation in position of the ligation. Therefore, not all 14-day group hearts had developed a thinned mature scar at this time point.

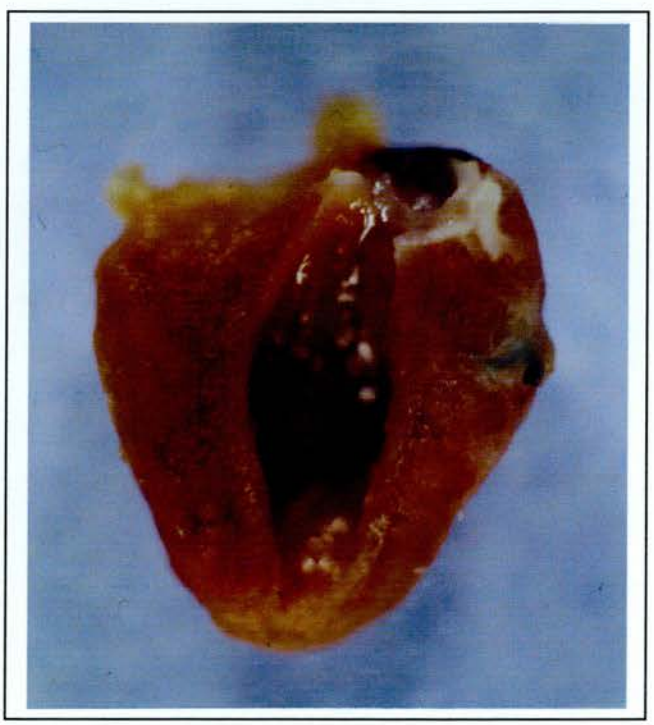
Gross analysis revealed hearts from rats in the antagonist treated group tended to have areas of increased myocardial content within the infarct at both time points. *Figure 4.1 (b)* depicts a cross-section of heart, cut apex to base, from a rat in the 14-day CAL + A-182086 group displaying a high level of 'salvaged' myocardium in the infarct. Furthermore, large vessels were present on the outer wall of some infarcts, indicating a blood supply to the salvaged tissue. Again, not all hearts in either the 7 or 14-day treated groups showed such a high level of salvaged myocardium within the infarct, with some hearts also displaying areas of thinning and normal scar formation within the infarct, similar to that observed in the CAL group.

Figure 4.1 Cross-section of hearts, cut apex to base, from both the (a) CAL and (b) CAL +A-182086 treated rat groups, 14 days post-MI.

(a)



(b)



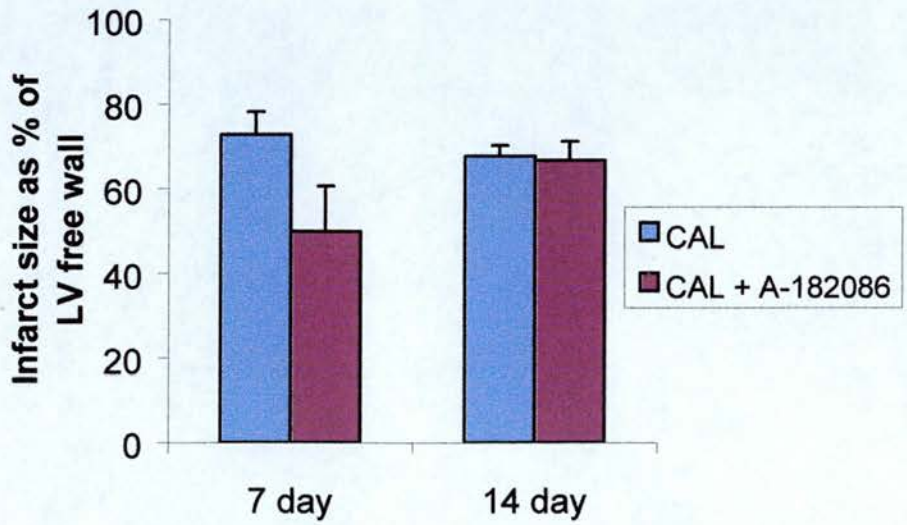
4.3.1.4 Infarct parameters

Infarct parameters were measured in both 7 and 14-day CAL and CAL + A-182086 groups, but not in the 2-day CAL or CAL + A-182086 groups as a defined infarct had not formed at this time point.

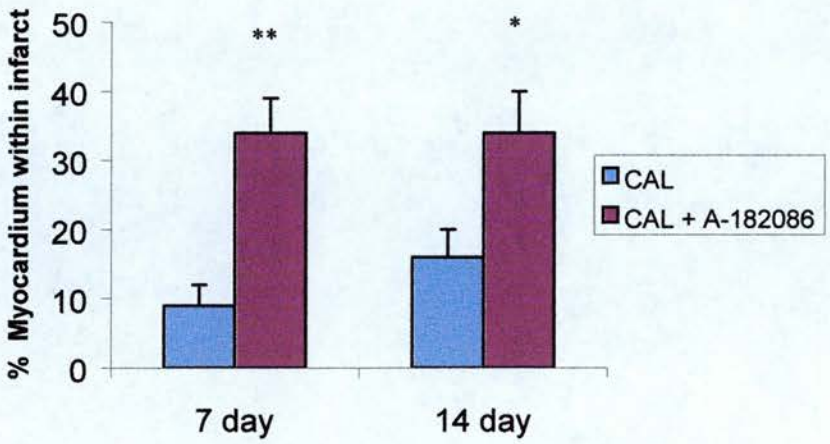
Within the 7 and 14-day groups, addition of antagonist did not alter infarct size (expressed as percentage of the LV free wall; *Figure 4.2 (a)*). However, the percentage of myocardium within the infarct (Figure *4.2 (b)*), and the myocardium to collagen content of the infarct (Figure *4.2 (c)*), expressed as a ratio, were significantly increased in the presence of A-182086 in both 7 ($P<0.01$) and 14-day ($P<0.05$) CAL-treated groups. Infarct thickness tended to be greater in the antagonist treated groups. However, significant differences were not observed (*Figure 4.2 (d)*).

*Figure 4.2 Effect of antagonist treatment on (a) infarct size as a % of the LV free wall; (b) % myocardial content within the infarct; (c) myocardial/collagen content within the infarct; and (d) infarct thickness as a % of respective CAL group, in the 7 and 14-day CAL groups. *P<0.05, **P<0.01 versus respective untreated group.*

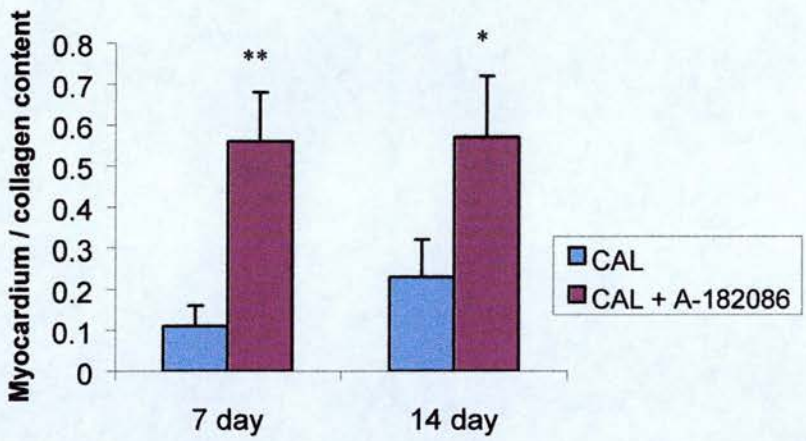
(a)



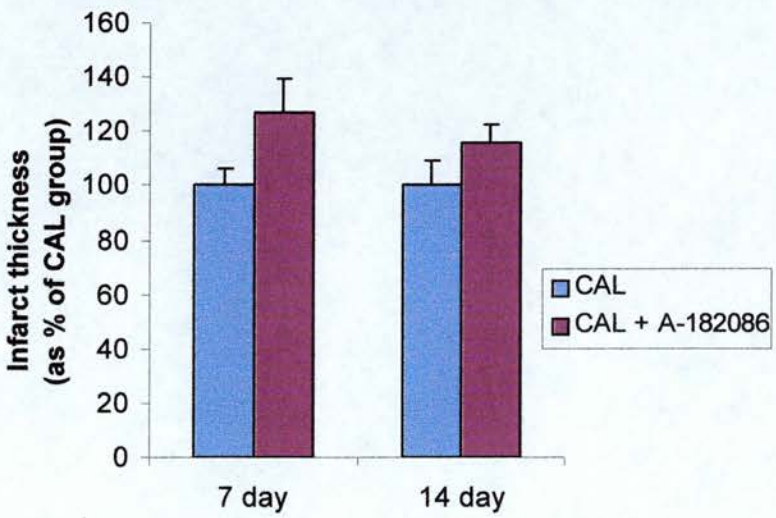
(b)


% Myocardium within infarct
50
40
30
20
10
0
**
*
7 day
14 day
CAL
CAL + A-182086

(c)


Myocardium / collagen content
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
**
*
7 day
14 day
CAL
CAL + A-182086

(d)


Infarct thickness
(as % of CAL group)
160
140
120
100
80
60
40
20
0
7 day
14 day
CAL
CAL + A-182086

4.3.2 Effect of CAL and A-182086 on the developing scar characterised using histological and immunohistochemical techniques.

Within the 2, 7 and 14-day CAL groups, van Gieson and neutrophil staining, GSL I, TGF-β_1 and ET-1 immunoreactivity within the infarct were all similar to that observed in the infarcts of rat hearts from the 2, 7 and 14-day CAL groups in Chapter 3 (sections 3.3.3.1-3).

4.3.2.1 2-day CAL + A-182086

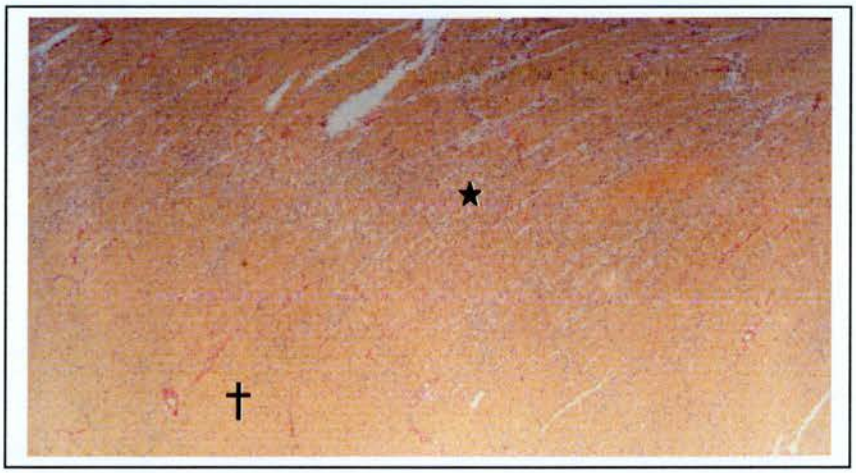
van Gieson's stain highlighted areas of necrosis interspersed with areas of viable myocardium throughout the infarct *(Figures 4.3 (a, b, c))* indicating that antagonist treatment tended to increase myocardium survival in the developing scar after MI at this time point. However, an increased accumulation of cells was observed in areas of necrotic tissue, most likely including proliferating fibroblasts, demonstrating that ET receptor antagonism did not significantly inhibit this occurrence.

An inflammatory cell response was observed in necrotic tissue within the infarct, with macrophages *(Figure 4.4 (a))* and neutrophils *(Figure 4.4 (b))* evident. However, the size of the area affected by inflammation reflected the size of tissue areas affected by the ligation. As areas of necrosis within the infarct tended to be less for the antagonist treated group, so also was the area of the inflammation. TGF-β_1 immunoreactivity in the non-infarcted LV and in salvaged myocardium within the infarct *(Figure 4.4 (c))* was increased compared to that observed in the RV, septum, or throughout myocardium in the sham or sham + A-182086 groups (not shown), indicating that ET receptor antagonism had no effect on increased TGF-β_1 staining of cardiomyocytes in these areas. Necrotic myocardium within the infarct showed faint TGF-β_1 staining *(Figure 4.4 (c))*.

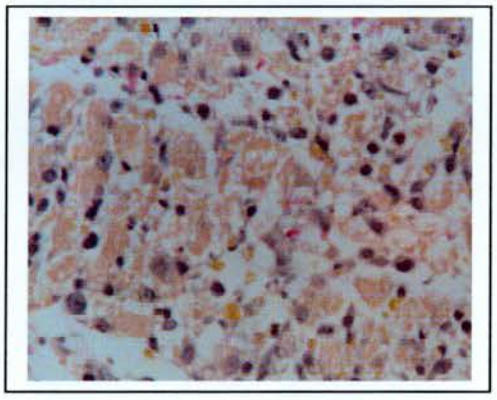
Areas of salvaged myocardial tissue within the infarct displayed ET-1 immunoreactivity similar to that observed in normal viable myocardium *(Figure 4.5 (a, c))*. Furthermore, ET-1 staining was observed in some macrophages, but few

Figure 4.3 Images of sections stained with van Gieson's stain from within the infarct, representative of hearts from the 2-day CAL + A-182086 group. (a) overview of area in the LV affected by ligation (x100 magnification); (b, ★) necrosis and increased cell content in areas affected by the ligation (x400 magnification); and (c, †) viable myocardium within the infarct (x400 magnification).

(a)



(b)



(c)

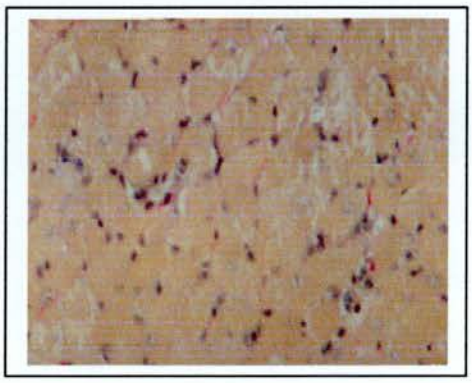
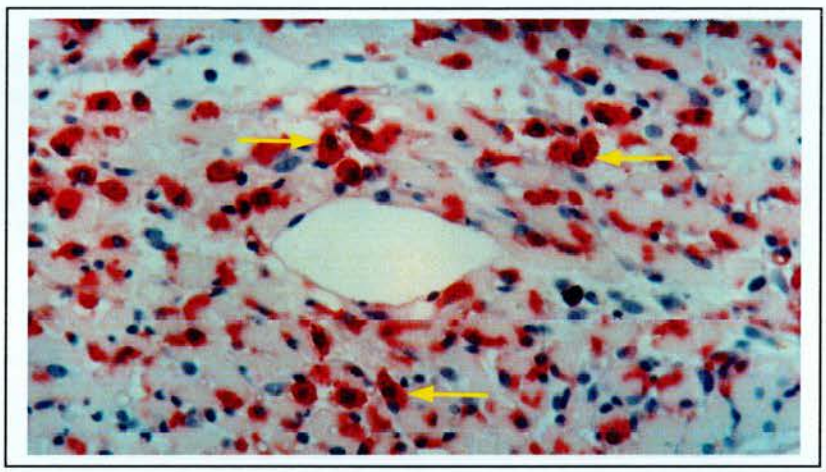
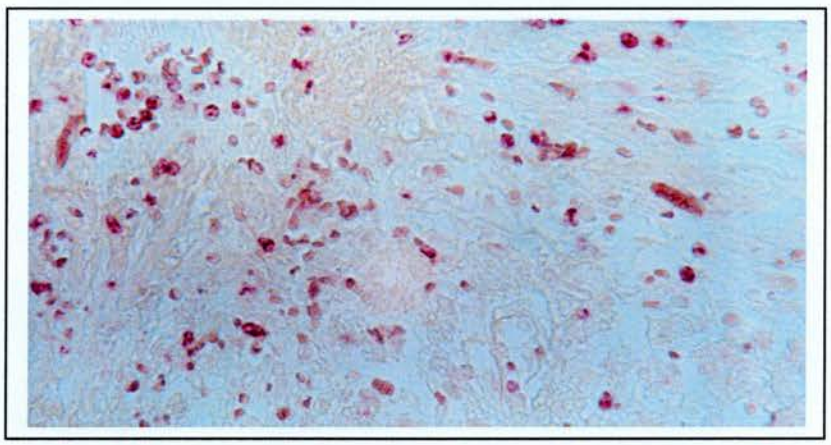


Figure 4.4 Images from within the infarct from sections representative of hearts from the 2-day CAL + A-182086 group. (a) GSL I immunoreactivity highlighting macrophages (arrows, x400 magnification); (b) neutrophil staining (x200 magnification); and (c) TGF-β_1 immunoreactivity (x100 magnification).

(a)



(b)



(c)

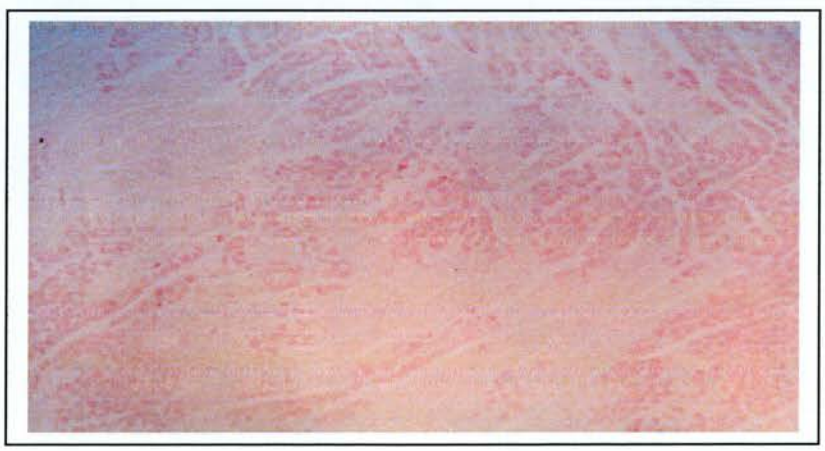
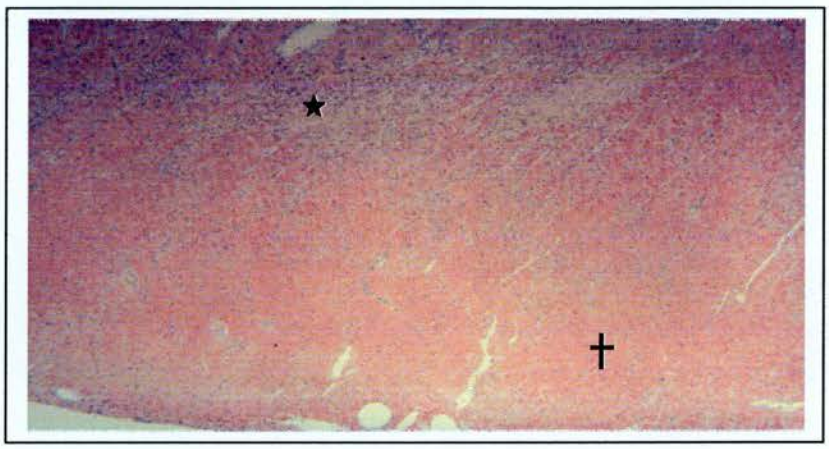
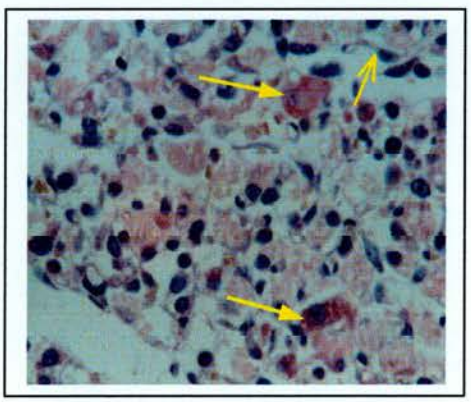


Figure 4.5 Images showing ET-1 immunoreactivity within the infarct of a heart representative of the 2 day CAL + A-182086 group. (a) overview (parallel to Figure 4.2(a)) of LV affected by ligation (x100 magnification); (b, ★) loss of staining in necrotic tissue and increased cell content including macrophages (closed arrowhead) and a fibroblast (open arrowhead, x400 magnification,); and (c,†) viable myocardium within the infarct (x200 magnification).

(a)



(b)



(c)

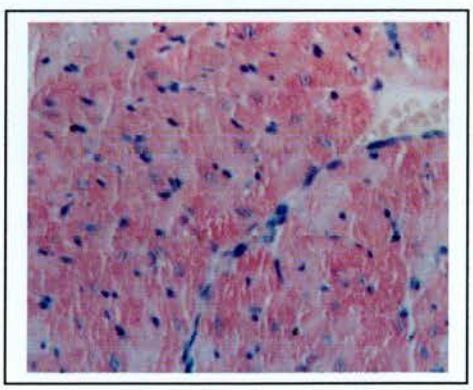
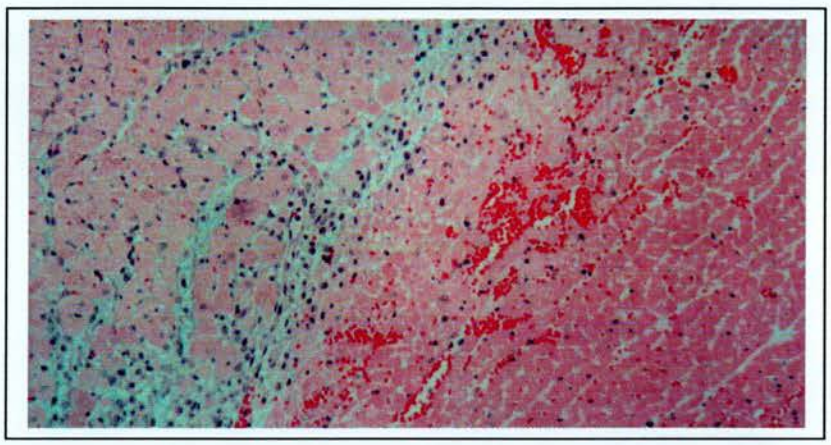
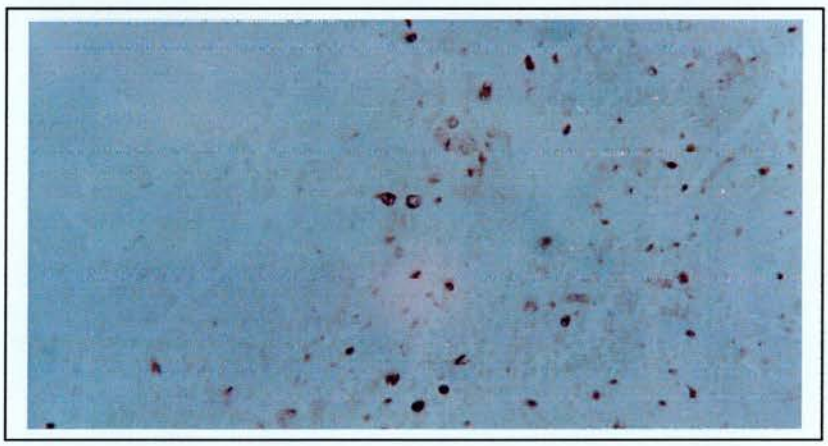


Figure 4.6 Images from the infarct border representative of hearts from the 2-day groups. (a) H&E staining (x400 magnification) and (b) parallel section TUNEL-positive staining (x400 magnification) at the infarct border of a heart from the 2-day CAL group compared to (c) TUNEL-positive staining (x400 magnification) at the infarct border of a heart from the 2-day CAL + A-182086 group.

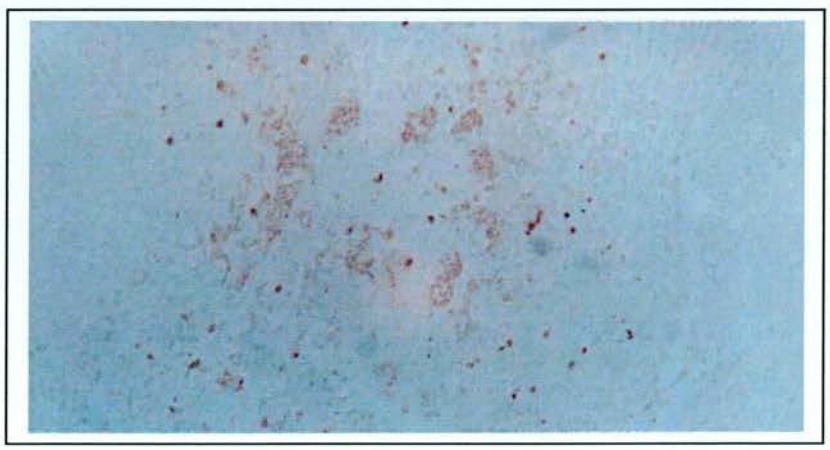
(a)



(b)



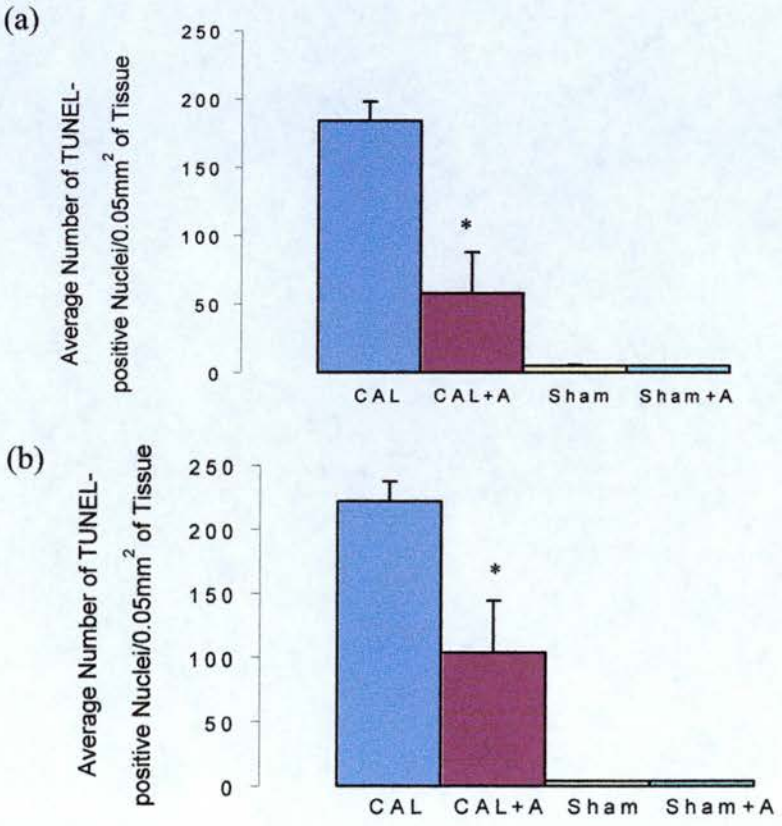
(c)



fibroblasts, within necrotic areas of myocardium *(Figure 4.5 (b))*. ET-1 staining was also faint in those cardiomyocytes within necrotic myocardium in the infarct *(Figure 4.5 (b))*.

Evidence of TUNEL positive labelling was investigated at both the border and central areas of the infarct and respective sham-op areas in rat hearts from all four 2-day groups. TUNEL positive cells (staining brown) identified in the infarct as described in section 2.2.3.4, were evident within central areas of the infarct and at the border (*Figure 4.6 (b)*). However, attempts to identify these cells using a parallel haematoxylin & eosin stained section from an identical area in the same heart *(Figure 4.6 (a))* proved extremely difficult. There was no evidence of TUNEL positive cells in the noninfarcted LV, RV or septum of CAL rats or in any areas of sham-op myocardium (not shown). Addition of A-182086 to the CAL group led to a significant reduction in TUNEL positive cell number at both the border *(Figure 4.6 (c), Figure 4.7 (a))* and central area *(Figure 4.7 (b))* of the infarct.

*Figure 4.7 Average number of TUNEL-positive nuclei per 0.05mm^2 of tissue at (a) the border and (b) in the centre of the infarct in the 4 treatment groups. *P<0.05 versus respective untreated group.*



4.3.2.2 7-day CAL + A-182086

At 7 days, administration of antagonist led to a significant increase in myocardial content within the infarct of hearts from CAL + A-182086 group rats. The salvaged myocardium showed a cellular morphology, vascular supply and myoendothelial distribution similar to that observed in normal or sham-op myocardium *(Figure 4.8 (a, c, d))*. However, where areas of granulation and necrosis were evident, fibroblasts were synthesising collagen, indicating that ET-1 is not essential in the regulation of this process (*Figure 4.8 (b)*).

As with the finding in the 2-day CAL + A-182086 group, the inflammatory response at 7 days tended to correlate with the level of necrotic damage present. The size of the area affected by inflammation in the antagonist treated group, therefore, tended to be less than in the untreated CAL group. Inflammatory cells evident in granulation areas included macrophages (*Figure 4.9 (a)*) and monocytes. New vessel formation was also present within areas of granulation tissue (*Figure 4.9 (b)*).

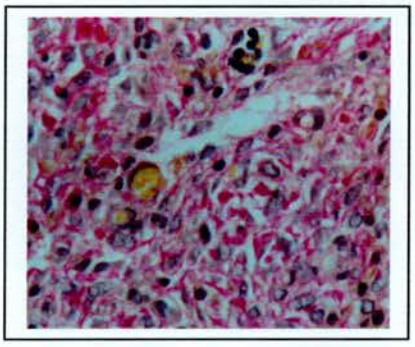
TGF-β_1 immunoreactivity was still evident in specific cells within areas of granulation in the infarct (*Figure 4.9 (c)*), comparable to that observed throughout the infarct of hearts in the respective untreated CAL group and also in the 7-day CAL group in Chapter 3. However, the number of TGF-β_1-stained cells again correlated with the amount of granulation tissue present. Therefore, in the antagonist treated CAL group, less TGF-β_1 stained cells were evident than in the untreated CAL group. In salvaged myocardium within the infarct, TGF-β_1 staining was greater than that observed in the RV or septum and similar to that observed in the non-infarcted LV (not shown).

ET-1 immunoreactivity in the salvaged myocardium was diffuse, with a distribution similar to that found in normal myocardium (*Figure 4.10 (a, c)*). Furthermore, in granulation tissue, ET-1 immunoreactivity was again located in fibroblasts and inflammatory cells (*Figure 4.10 (b)*), and in endothelial cells of newly forming vessels (not shown). Intensity of staining in these cells was similar to that observed

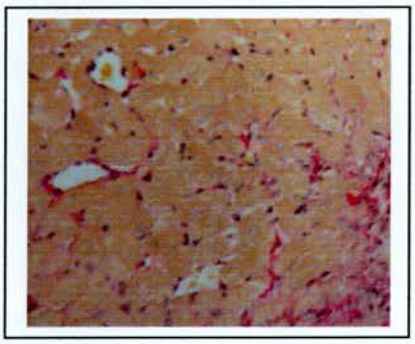
Figure 4.8 Images from within the infarct, representative of heart sections from the 7 day CAL + A-182086 group, stained with van Gieson's stain. (a) overview of the LV area affected by ligation (x50 magnification); (b, ★) collagen synthesis in granulation tissue (x400 magnification); (c, †) salvaged myocardium within the infarct (x400 magnification); and (d, †) salvaged myocardium showing a normal myoendothelial cell distribution (x400 magnification).

(a)

(b)



(c)



(d)

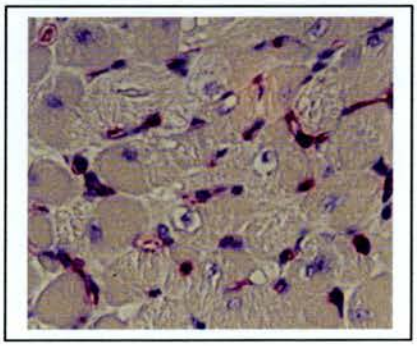
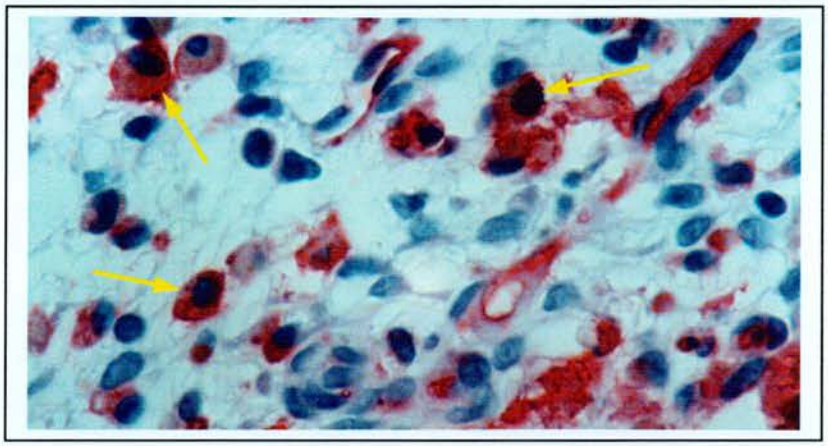
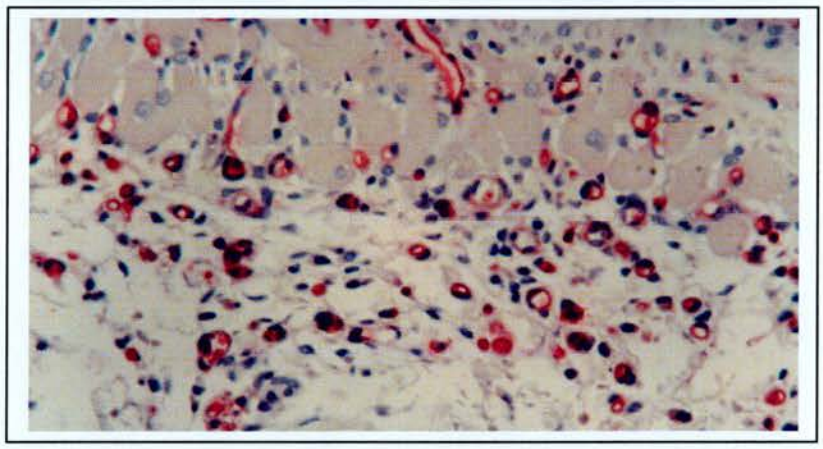


Figure 4.9 Images from within granulation tissue of the infarct from heart sections representative of the 7-day CAL + A-182086 group. GSL I immunoreactivity highlighting (a) inflammatory cells including macrophages (arrows; x1000 magnification), and (b) new vessel formation (x400 magnification); (c) TGF-β_1 immunoreactivity in inflammatory cells (closed arrowhead), fibroblasts (open arrowhead) and in endothelia of new vessels (dotted arrow line; x400 magnification).

(a)



(b)



(c)

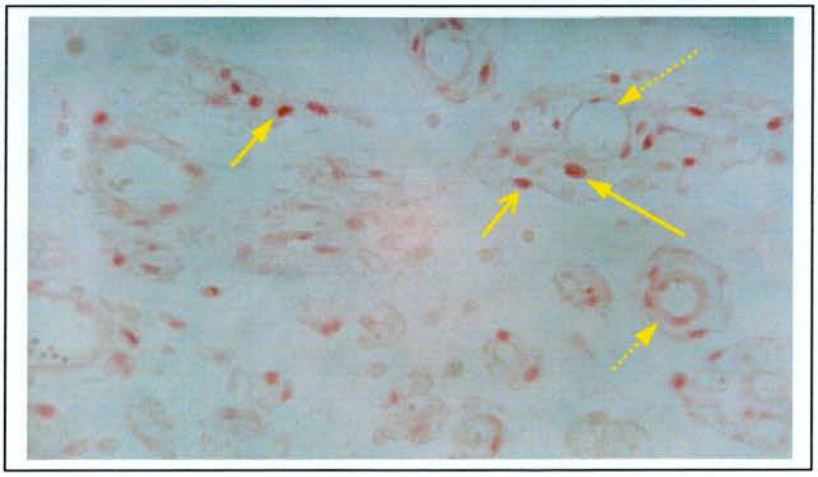
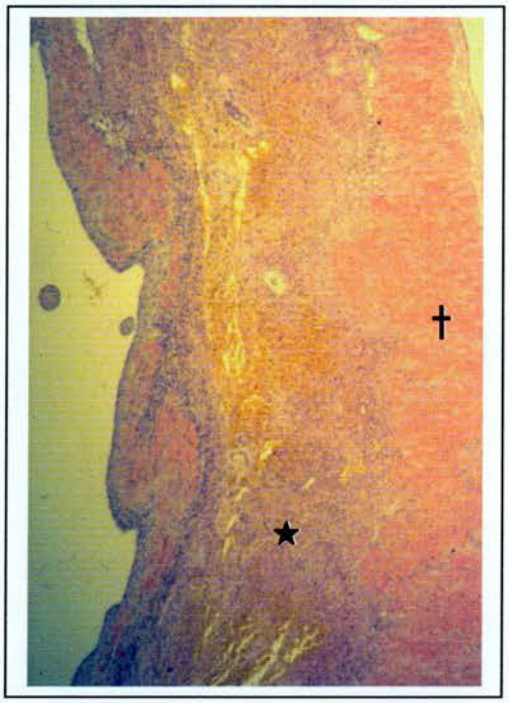
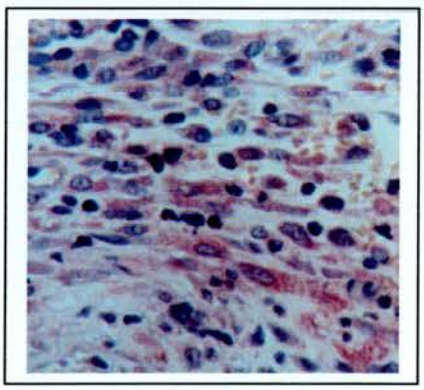


Figure 4.10 Images from within the infarct, representative of heart sections from the 7 day CAL + A-182086 group, showing ET-1 immunoreactivity. (a) overview of the LV area affected by ligation (x50 magnification); (b, ★) ET-1 staining in granulation tissue (x400 magnification); and (c, †) salvaged myocardium within the infarct showing ET-1 immunoreactivity (x400 magnification).

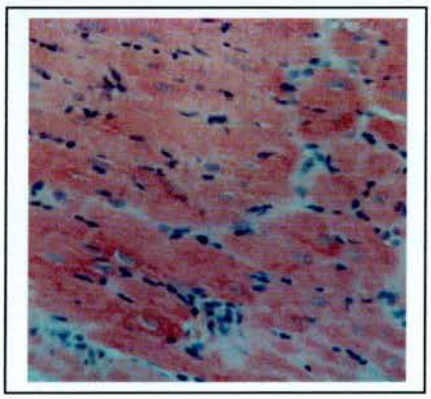
(a)



(b)



(c)



in cells within the infarcts of hearts from untreated CAL group rats. ET-1 immunoreactivity was again observed in cells that showed TGF-β_1 immunoreactivity.

4.3.2.3 14-day CAL + A-182086

The significantly increased myocardial cell content within the infarcts of hearts from the 14-day CAL + A-182086 group rats (*Figure 4.11 (a)*), compared to the untreated CAL group alone, again had a cellular morphology, vascular supply and ET-1 immunoreactivity (*Figure 4.11 (b)*) similar to normal myocardium. The salvaged myocardium also showed a myoendothelial capillary distribution comparable to that observed in normal or sham-op myocardium (not shown).

Increased TGF-β_1 immunoreactivity was again evident in cardiomyocytes in the non-infarcted LV and in salvaged myocardium within the infarct (not shown). Furthermore, mature collagen deposition, TGF-β_1 staining, remaining inflammatory cells and new vessel formation were all observed in areas where granulation tissue was still present (not shown).

β-MHC immunoreactivity was increased in the non-infarcted LV and in remaining viable cardiomyocytes within the infarct of hearts from CAL group rats (*Figure 4.12. (b)*) compared to sham-op group rats (*Figure 4.12 (a)*). However, addition of A-182086 to the CAL group tended to reduce β-MHC immunoreactivity in most sections in both the non-infarcted LV (*Figure 4.12 (c)*) and in the salvaged myocardium within the infarct.

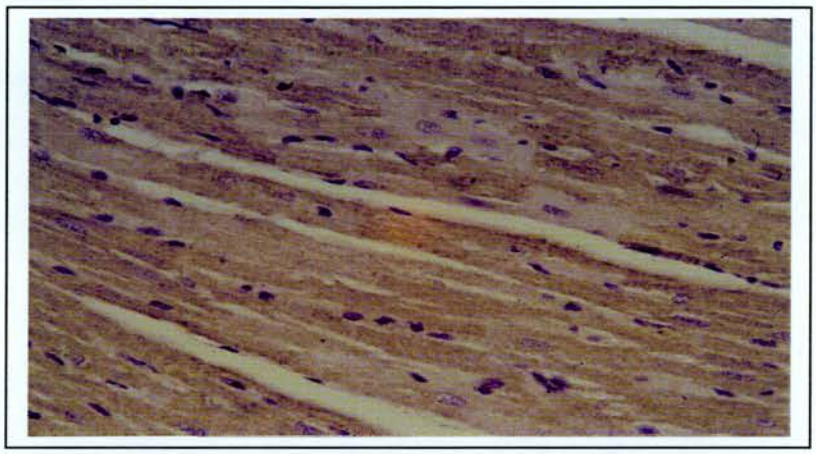
Figure 4.11 Images from within the infarct of a heart from the 14-day CAL + A-182086 group. Overview of the infarct stained with (a) van Gieson's stain (x50 magnification) and (b) showing ET-1 immunoreactivity in a parallel section (x50 magnification).

(a)

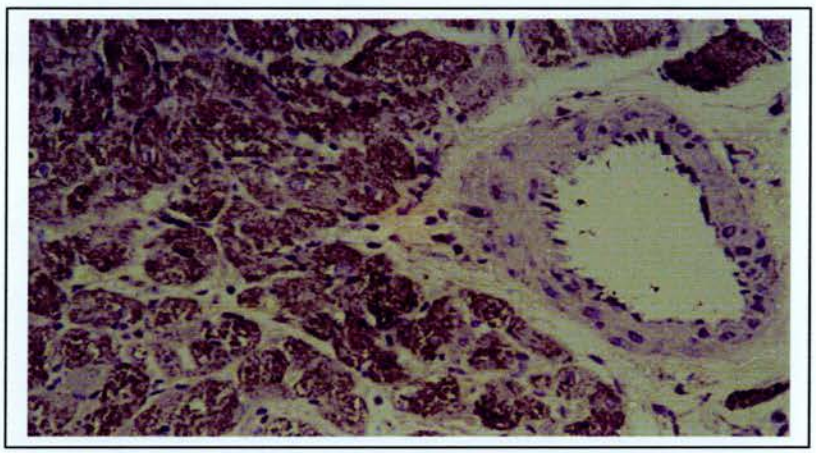
(b)

Figure 4.12 Images showing β-MHC immunoreactivity (brown) in the non-infarcted LV of hearts representative of the 14-day groups. (a) sham-op; (b) CAL; and (c) CAL + A-182086 groups (all x400 magnification).

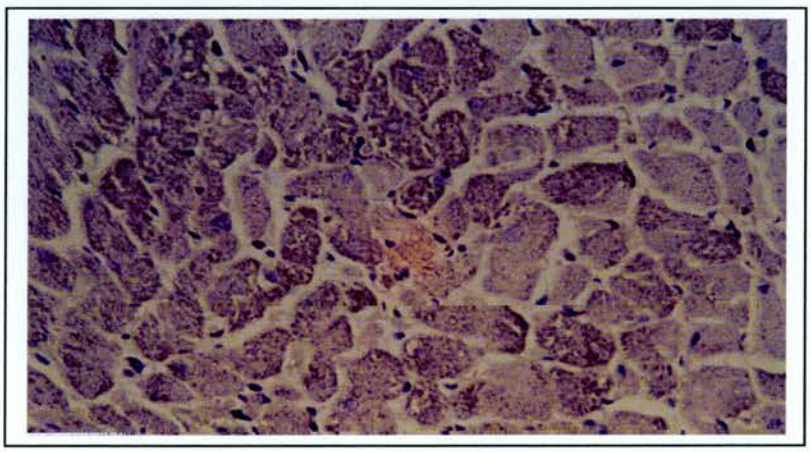
(a)



(b)



(c)



4.4 Discussion

This study set out to investigate the role of ET-1 within the developing scar of rat hearts in the early stage post-MI, using a non-selective ET receptor antagonist A-182086 (Abbott Laboratories, USA). In A-182086-treated and untreated CAL rats, MI was confirmed by an increased plasma LDH value, measured upon recovery from surgery, 3x that of a sham rat operated on the same day. Verification that the antagonist sufficiently blocked ET_B receptors was indicated by a significantly increased plasma ET-1 value in all antagonist-treated groups compared to their respective untreated groups; the ET_B receptor has been shown to be associated with clearance of ET-1 from plasma (Fukuroda *et al.,* 1994).

Results from this study indicated that oral administration of A-182086 significantly decreased heart weight at 14 days post-CAL and significantly improved myocardial cell content within the infarct at both 7 and 14 days post-CAL. The salvaged myocardium showed a cellular morphology, vascular and myoendothelial supply, and distribution of ET-1 immunoreactivity similar to that found in normal or sham-op myocardium.

Although ET-1 immunoreactivity was increased within the infarcts of both the CAL and CAL + A-182086 treated group at 7 and 14 days, it was not known (at the start of the present study) if preproET-1, ET_A or ET_B receptor mRNA levels change in conjunction with the increase in ET-1 immunoreactivity observed. Also, if changes were observed, would administration of antagonist have an effect on expression levels in either the infarct or non-infarcted myocardium of the LV? However, attempts to investigate mRNA levels using *in situ* hybridisation in this thesis study were not successful (see section 4.2.4). Recently, though, Tønnessen *et al.* (1998) demonstrated a 3.5 ±1.1-fold and a 6.4 ±0.8-fold upregulation of preproET-1 mRNA in the noninfarcted and infarcted area of the left ventricle respectively at 7 days post-MI in the rat, indicating an increase in expression parallel to the increased immunoreactivity within the infarct. Furthermore, another study reported significantly increased preproET-1 mRNA expression in the LV of hearts from rats

15 days post-MI, with administration of bosentan (24 hours after CAL) having no effect on preproET-1 mRNA levels (Øie *et al.,* 1998). This finding by Øie *et al.* is consistent with the finding of increased ET-1 immunoreactivity within the infarct being unaffected by non-selective ET receptor antagonism in the present study, suggesting ET receptor blockade does not inhibit synthesis of cellular ET-1 in the developing scar.

Other factors, including Ang II and specific cytokines, have been shown to stimulate ET-1 synthesis in both endothelial cells (Kanse *et al.,* 1991) and fibroblasts (Gray *et al.,* 1998). ACE inhibitors were shown to suppress both preproET-1 mRNA overexpression in the kidney of a rat model of immune-complex nephritis (Ruiz-Ortega *et al.,* 1997), and reduce endogenous ET-1 secretion, resulting in improved coronary function and stabilisation of cardiac rhythm after ischaemia in a perfused rat heart model of ischaemia/reperfusion (Brunner & Opie, 1996). Furthermore, TGF-β_1 was found to stimulate the expression of preproET-1 mRNA by vascular endothelial cells (Kurihara *et al.,* 1989). The increased ET-1 immunoreactivity observed in specific cells at 7 days within the infarct of treated and untreated groups may, therefore, be a consequence of paracrine stimulation of synthesis from such factors as Ang II and cytokines. It would be interesting to investigate the action of the upregulated RAAS on ET-1, ET_A and ET_B mRNA expression, using an AT_1 receptor antagonist administered immediately post-MI in the rat, therefore determining the influence of increased Ang II on the ET system during early scar development.

A-182086 significantly improved myocardial content within the infarct at both 7 and 14 days post-CAL. However, where areas of granulation were present within the developing scar, active processes of scar formation including inflammatory cell infiltration, collagen synthesis, angiogenesis, and TGF-β_1 synthesis were still present and not inhibited by non-selective ET receptor antagonism. Therefore, though A-182086 did reduce the amount of damaged tissue within the infarct affected by the ligation, it did not inhibit the processes of scar healing in areas of granulation tissue. This suggests that though increased ET-1 immunoreactivity was observed within the

developing scar and may contribute to regulation of the above-mentioned processes, blockade of its effect was insufficient to halt the scaring process.

Factors other than ET-1, including the RAAS and cytokines, have also been reported to be increased within the infarct early after MI and can influence scar development. Levels of renin mRNA were found to be increased 4-, 14-, and 8-fold in the infarcted LV at 2, 7 and 14 days respectively after MI in rats, suggesting a role for intracardiac Ang II in the infarct healing process (Passier *et al.,* 1996). Activation of the renin gene in the border zone of the infarcted area in the early phase after MI was also in agreement with the activation and localisation of the ACE gene and protein (Passier *et al.,* 1995). Observations of increased interstitial collagen deposition and the effect of ACE-inhibitors resulted in the hypothesis that the RAAS might be involved in the regulation of collagen synthesis in the cardiac interstitium following MI (Michel *et al.,* 1988). Recent AT receptor antagonist studies have also shown Ang II to be involved in both interstitial cell proliferation (via the AT_2 receptor) and collagen deposition (via the AT_1 receptor) in rats (Unger *et al.,* 1998; Kuizinga *et al.,* 1998). Furthermore, the ACE inhibitor captopril and the AT_1 receptor antagonist valsartan were shown to have potent inhibitory effects on pro-inflammatory cytokines including TNF-α and IL-1 *in vitro,* suggesting a role for the RAAS in regulation of pro-inflammatory factors (Peeters *et al.,* 1998). Ang II has also been demonstrated to stimulate angiogenesis in a murine sponge model of angiogenesis (Machado *et al.,* 1999).

Cytokine gene expression, including TNF-α, IL-1β, and IL-6, has been reported to be increased after MI in rat hearts (Ono *et al.,* 1998) and may have predominant roles in regulating inflammatory responses in the infarcted myocardium (Frangogiannis *et al.,* 1998). Induction of cytokine expression was also found in leukocytes in acute MI in an ischaemia/reperfusion rat heart model (Marx *et al.,* 1997). TGF-β_1 is an important regulatory peptide in fibrous tissue formation and has numerous actions on extracellular matrix. It stimulates fibroblast-like cell growth, enhances collagen synthesis, and suppresses collagen degradation (O'Kane *et al.,* 1997). Furthermore, TGF-β_1 mRNA has been shown to be increased in necrotic tissue within the infarcted

rat heart after MI, with levels peaking at day 7 (Sun *et al.,* 2000), supporting a role in the processes of scar formation. The above factors may, therefore, be equally important regulators of scar formation in the early stage post-MI, and may continue the reparative processes independent of the presence or influence of ET-1.

As reported in section 3.3.2, increased ET-1 and TGF-β_1 immunoreactivity was observed in similar cells within the infarct at 7 days post-CAL, although it is not known if ET-1 has a predominant role in regulating TGF-β_1 synthesis or vice versa. However, administration of A-182086 did not prevent increased TGF-β_1 cell specific immunoreactivity in areas of granulation at this time point, suggesting ET-1 is not essential in regulation of this peptide. This is in contrast to a recent study showing significantly reduced TGF-β_1 mRNA expression and peptide levels in the LV of a rat MI model in the presence of bosentan (administered 24 hours post-CAL), further suggesting that ET-1 may contribute to fibrosis early post-MI via activation of TGF-β_1 within the myocardium (Tzanidis & Lim, 1999). However, this previous study did not discriminate between levels of TGF-β_1 in the infarct and non-infarcted LV. Therefore, this finding may not be dissimilar to the finding in the present study, where overall distribution of TGF-β_1 immunoreactivity tended to be less in the antagonist treated group, since less granulation tissue was present within the infarct.

The fact that A-182086 did not inhibit synthesis of TGF-β_1 in the developing scar suggests that other factors present in the infarct may also stimulate TGF-β_1 synthesis. Ang II has been reported to specifically stimulate TGF-β_1 expression in the developing infarct (Sun *et al.,* 1998), and as mentioned earlier, the RAAS itself is upregulated in the early stage post-MI. Nevertheless, as A-182086 did not decrease TGF-β_1 staining distribution in granulation tissue or cardiomyocytes, TGF-β_1 may alternatively have a predominant role in stimulation of ET-1 synthesis in the developing scar. TGF-β_1 has been reported to stimulate ET-1 mRNA expression in vascular endothelial cells *in vitro* (Kurihara *et al.,* 1989), and upregulate ET-1 and big ET-1 peptide secretion in a human prostate cancer cell line *in vitro* (Lechat *et al.,*

1999). However, whether TGF-β_1 has similar effects *in vivo,* and within the developing scar, requires further investigation.

Neutrophil infiltration and expression of neutrophil adhesion molecules have been shown to correlate with myocardial infarct size (Hillis *et al.,* 1999), with rats deficient in neutrophil adhesion molecules P-selectin, E-selectin, ICAM-1 or CD11/CD18 all showing reduced myocardial damage in the LV after ischaemia/ reperfusion (Trocha *et al.,* 1999). Though ET-1 has been implicated in stimulating neutrophil adherence during coronary artery bypass (Bugajski *et al.,* 1999), and adherence to coronary artery endothelial cells *in vitro* (Zouki *et al.,* 1999), in the present study many neutrophils were still observed in the infarct in the presence of A-182086 indicating that blockade of ET receptors is insufficient to inhibit neutrophil infiltration early post-MI. However, many other factors can also stimulate neutrophil adhesion and infiltration including cytokines (Sawa *et al.,* 1998), complement, and oxygen free radicals released from hypoxic cardiomyocytes (reviewed by Jordan *et al.,* 1999). Again, these factors may stimulate neutrophil infiltration and activation independent of the presence of ET-1.

A modified *in situ* nick end-labelling (TUNEL) assay (Gavrieli *et al.,* 1992) was used for assessment of apoptosis in myocardial tissue within the infarct. TUNEL-positive staining has been shown to increase during the first 6 hours to 2 days after permanent coronary occlusion (Kajstura *et al.,* 1996), with levels decreasing over 4 weeks (Cheng *et al.,* 1996). Within the 2-day CAL groups, levels of TUNEL-positive cells at both the border and within the central area of the infarct were significantly reduced in the presence of A-182086. However, interpretation of these results is complicated. TUNEL-positivity reflects a wide range of cellular conditions including viable cells undergoing repair, necrosis and apoptosis. Although TUNEL-positivity is not the best hallmark of apoptosis, there is presently no other way to semi-quantitatively assess apoptosis in myocardial tissue (Yaoita *et al.,* 2000). To confirm apoptosis, TUNEL-positive cells would need to be examined via electron microscopy to reveal condensation and fragmentation of the nucleus, which are hallmarks exclusive to apoptosis (Yaoita *et al.,* 2000).

Attempts to identify TUNEL-positive cells using parallel haematoxylin & eosin stained sections proved extremely difficult. Furthermore, whether antagonist administration induced a direct inhibition of TUNEL-positive stained cells, or indirectly reduced the number of infiltrating inflammatory cells and resident cells affected by ischaemia, therefore leading to a reduction in numbers of cells undergoing TUNEL-positive staining, could not be determined. However, ET receptor antagonism did significantly reduce levels of TUNEL-positive staining within the infarct suggesting ET-1 may have a role in regulating apoptosis post-MI. No studies to date have investigated the effect of ET-1 on apoptosis post-MI. One study showed TUNEL-positive staining cells within the infarct after MI included cardiomyocytes, endothelial cells, macrophages and neutrophils (Takemura *et al.,* 1998), all of which have been shown to stain positive for ET-1 immunoreactivity in the present study. However, further studies verifying apoptosis using electron microscopy, and using double staining techniques along with the TUNEL assay to identify specific cells (Willingham *et al.,* 1999), are required to clarify whether ET-1 has a role in regulating apoptosis post-MI.

In summary, ET receptor antagonism did not halt the active processes of scar formation within the infarct including the inflammatory response, angiogenesis, collagen synthesis, or activation of TGF-β_1. Nevertheless, administration of A-182086 did significantly improve viable myocardial content of the infarct in both 7 and 14-day CAL-treated groups. The fact that areas of inflammation and increased cellular content were still evident in the infarct at 2 days post-CAL suggests that the primary action of antagonist treatment in increasing levels of salvaged myocardium is manifest at a time earlier than 2 days. Furthermore, 2 CAL + A-182086 group rats (1 from the 7-day group and 1 from the 14-day group) that did not eat their food over the first 24 hours, but ate daily thereafter for the duration of the study, developed thinned defined infarcts with little myocardial cell content. This further implies that the antagonist effect was occurring within the first 24 hours of treatment. Neither rats were included in the analysis of these studies.

The question therefore arises as to how early administration of A-182086 leads to an improvement in myocardium content within the infarct. The answer may involve whether myocardial ET-1 tissue levels change acutely after irreversible myocardial damage. Increased plasma ET-1 concentrations were found in patients with unstable angina, myocardial infarction (Wieczorek *et al.,* 1994) and immediately following percutaneous transluminal coronary angioplasty (Malatino *et al.,* 1993). Hypoxia is also reported to induce ET gene expression and secretion in cultured human endothelial cells *in vitro* (Kourembanas *et al.,* 1991); and increase maximal responses of ET-1 in the rat isolated perfused mesenteric arterial bed *in vitro* (Douglas *et al.,* 1991).

Although studies investigating acute changes in tissue levels of ET-1 in the heart after induced ischaemia *in vitro* are few in number, studies investigating ET-1 during ischaemia/reperfusion *in vitro* have reported an increased release of endogenous ET-1 in the coronary perfusate (Brunner *et al.,* 1992) and beneficial effects of ET receptor antagonism on ischaemia/reperfusion-induced myocardial damage (Pernow & Wang, 1997). Literature investigating acute changes in ET-1 in the heart after induced ischaemia *in vivo* found increased ET-1 expression and production of ET-1 in cardiomyocytes (Tønnessen *et al.*, 1995). It is therefore possible that stimulation of an acute release of ET-1 as a consequence of irreversible hypoxic damage in the myocardium may potentiate a profound transient vasoconstriction, further reducing any remaining blood flow around (or into) the affected tissue, thereby augmenting irreversible ischaemic damage. Evidence of a collateral flow has been reported in the rat heart during myocardial ischaemia (Maxwell *et al.,* 1987) and in human studies, where coronary collateral circulation decreased wall motion abnormalities, ST segment changes and lactate production post-MI (reviewed by Charney & Cohen, 1993). Although transient increases in other vasoconstrictive factors may also occur during this period, ET-1, being one of the most potent vasoconstrictive peptides known, may be responsible for most of the damage induced. Therefore, inhibition of a transient ET-1-mediated vasoconstriction in the acute phase post-MI may explain how early administration of A-182086 improved myocardial survival within the infarct while not blocking the later processes of scar formation.

Recent antagonist studies have allowed further insight into the role of ET-1 in the heart post-MI. An interesting study by Hu *et al.* (1998) reported that ET_A selective receptor antagonism administered 3 hours after coronary artery ligation in rats and continued for 8 weeks aggravated chronic LV remodelling with no improvement in hemodynamics. Another recent study by Galuppo *et al.* (1999) also found early ET_A receptor blockade (started 3 hours after CAL) promoted infarct thinning and expansion in a rat model of MI. The fact that immediate administration of the ET_A receptor antagonist did not improve cardiac parameters in either study (change in myocardial cell content within the infarcts was not mentioned) suggests that selective blockade of ET_A receptors does not completely inhibit ET-1 mediated effects (such as vasoconstriction) early post-MI. ET_B receptor-mediated pathways are normally related to induction of vasodilation by enhancement of endothelial nitric oxide synthesis. However, ET_B receptors, present on vascular smooth muscle cells and cardiomyocytes (Suzuki *et al.,* 1993), can also contribute to the vasoconstrictor effect of ET-1 (McMurdo *et al.,* 1993; Seo *et al.,* 1994). In a study investigating ischaemia /reperfusion in the pig, both ET-1 and Ala-ET-1, a selective ET_B agonist, caused coronary vasoconstriction in the post-ischaemic myocardium (Wang *et al.,* 1995). Furthermore, Zhang *et al.* (1998) reported coronary vasoconstriction during and after perfusion with the ET_A selective antagonist BQ-123 in isolated Langendorff rat hearts, but not with the non-selective ET receptor antagonist bosentan, indicating ET_B receptor-mediated vasoconstriction in the presence of ET_A receptor antagonism. Therefore, antagonism of both ET receptors may be required to inhibit any transient ET-1 induced vasoconstriction during ischaemia.

Timing of antagonist administration also seems to be crucial. ET_A receptor antagonism administered 24 hours post-MI and continued for 4 weeks led to thinning of the LV scar, LV dilatation and an increase in LVEDP, with an improvement in RVSP and RAP (Picard *et al.,* 1998). Furthermore, administration of bosentan 24 hours post-MI and continued for 15 days did not reduce hypertrophy in the noninfarcted LV, though a modest reduction of preload and afterload provided by bosentan substantially attenuated LV dilatation causing improved pressure volume relationships (Øie *et al.,* 1998). However, bosentan administered 3 hours post-

ligation in the rat and continued for 8 weeks had no effect on LVEDP, but reduced SBP, HR and ventricular ET-1 content (Fraccarollo *et al.,* 1997). None of the studies alluded to the effect of antagonist treatment on the processes of scar formation or myocardial content within the scar.

Therefore, the finding that early ET_A receptor antagonism in CAL-operated rats (Hu *et al.,* 1998; Galuppo *et al.,* 1999) did not produce similar findings to the present study may be due to the potential role of ET_B receptors in ET-1 mediated vasoconstriction during ischaemia. Furthermore, the fact that bosentan administered 24 hours post–MI did not reduce LV hypertrophy (Øie *et al.,* 1998) along with the finding in the present study that A-182086 administered upon recovery from CAL surgery reduced heart weight, β-MHC immunoreactivity and improved myocardial content within the infarct also suggests that timing of antagonist administration, and therefore early transient changes in myocardial ET-1 levels may be crucial in the processes of tissue recovery and the extent of ischaemia-induced myocardial damage. It would, therefore, be interesting, in a future study, to compare the effect on myocardial content and scar formation after 14 days in (1) rats administered a non-selective ET receptor antagonist for the first 24 hours only post-CAL; (2) rats not fed antagonist in the first 24 hours post-CAL but treated daily thereafter for the duration of the study; and (3) rats fed antagonist during the first 24 hours post-CAL and continued daily for the 14 day period. This would confirm if treatment was effective over the first 24-hour period only, or required throughout the duration of scar formation.

Although myocardial content within the infarct was improved in the 7- and 14-day treated groups, there were no significant improvements in MAP or LVEDP at these time points. Also, it is not clear if the increased myocardial content observed will have beneficial or detrimental effects on the heart at later points during the progression towards CHF. Further studies are therefore required using the same protocol to investigate if chronic A-182086 treatment would improve cardiac parameters and/or remodelling during development of CHF. One study investigating chronic non-selective ET receptor antagonism (administered 3 hours after CAL in the

rat) also found no hemodynamic improvements at 7 days post-CAL. However, after 8 weeks bosentan treatment, left ventricular dilatation was partially prevented and improved hemodynamics were observed, supporting a role for ET-1 in left ventricular remodelling after MI (Fraccarollo *et al.,* 1997). Other studies have reported that long-term treatment with either bosentan (administered 7 days post-MI; Mulder *et al.,* 1997) or SB209670 (administered 10 days post-MI; Sakai *et al.,* 2000) markedly increased survival in the rat model of CHF. This increase in survival was associated with decreases in both preload and afterload and an increase in CO as well as decreased LV hypertrophy, LV dilatation, and cardiac fibrosis (Mulder *et al.,* 1997); and also an improvement in alterations of cardiac genes associated with progression of CHF (Sakai *et al.,* 2000).

Administration of A-182086 did significantly reduce heart weight in the 14-day CAL group, which was accompanied by reduced β-MHC immunoreactivity within the non-infarcted LV and in the salvaged myocardium within the infarct at this time point. Isoforms of cardiac MHC are altered in the failing myocardium of experimental CHF animals. Furthermore, the change from α-MHC to β-MHC is regarded as a molecular marker for hypertrophy in the failing myocardium (Michel *et al.,* 1988; Parker *et al.,* 1990b). ET-1 has also been shown to stimulate β-MHC production in ventricular myocardium *in vitro* (Wang *et al.,* 1992; Ichikawa *et al.,* 1996). That β-MHC immunoreactivity tended to be reduced in the non-infarcted LV and salvaged myocardium within the infarct of rat hearts from the A-182086 treated group suggests that early intervention with the non-selective ET receptor antagonist may have beneficial effects on wall stress, induction of hypertrophy, and LV remodelling at later stages post-MI. However, actual tissue levels of β-MHC would need to be measured in this study to confirm immunohistochemical observations. Interestingly, Øie *et al.* (1998) found no effect of bosentan treatment on LV hypertrophy 15 days post MI, with treatment started 24 hours post-CAL. This finding, combined with that from this thesis and that above of Fraccarollo *et al.* (1997) and Mulder *et al.* (1997), suggests that chronic ET antagonism may have both a direct effect on hypertrophy and remodelling at later stages post-MI, but also an indirect effect via an overall increase in viable tissue, and therefore a reduced work

rat) also found no hemodynamic improvements at 7 days post-CAL. However, after 8 weeks bosentan treatment, left ventricular dilatation was partially prevented and improved hemodynamics were observed, supporting a role for ET-1 in left ventricular remodelling after MI (Bauersachs *et al.,* 1998). Other studies have reported that long-term treatment with either bosentan (administered 7 days post-MI; Mulder *et al.,* 1997) or SB209670 (administered 10 days post-MI; Sakai *et al.,* 2000) markedly increased survival in the rat model of CHF. This increase in survival was associated with decreases in both preload and afterload and an increase in CO as well as decreased LV hypertrophy, LV dilatation, and cardiac fibrosis (Mulder *et al.,* 1997); and also an improvement in alterations of cardiac genes associated with progression of CHF (Sakai *et al.,* 2000).

Administration of A-182086 did significantly reduce heart weight in the 14-day CAL group, which was accompanied by reduced β-MHC immunoreactivity within the non-infarcted LV and in the salvaged myocardium within the infarct at this time point. Isoforms of cardiac MHC are altered in the failing myocardium of experimental CHF animals. Furthermore, the change from α-MHC to β-MHC is regarded as a molecular marker for hypertrophy in the failing myocardium (Michel *et al.,* 1988; Parker *et al.,* 1990b). ET-1 has also been shown to stimulate β-MHC production in ventricular myocardium *in vitro* (Wang *et al.,* 1992; Ichikawa *et al.,* 1996). That β-MHC immunoreactivity tended to be reduced in the non-infarcted LV and salvaged myocardium within the infarct of rat hearts from the A-182086 treated group suggests that early intervention with the non-selective ET receptor antagonist may have beneficial effects on wall stress, induction of hypertrophy, and LV remodelling at later stages post-MI. However, actual tissue levels of β-MHC would need to be measured in this study to confirm immunohistochemical observations. Interestingly, Øie *et al.* (1998) found no effect of bosentan treatment on LV hypertrophy 15 days post MI, with treatment started 24 hours post-CAL. This finding, combined with that from this thesis and that above of Fraccarollo *et al.* (1997) and Mulder *et al.* (1997), suggests that chronic ET antagonism may have both a direct effect on hypertrophy and remodelling at later stages post-MI, but also an indirect effect via an overall increase in viable tissue, and therefore a reduced work

load on remaining myocardium within the LV, when administered early post-MI. However, the precise mechanisms of how ET receptor antagonism may reduce hypertrophy directly, and indirectly, remain to be clarified.

Chapter 5

Investigation of the myocardial endothelin system 5 & 12 weeks post myocardial infarction.

5.1 Introduction

In response to myocardial infarction (MI) compensatory mechanisms are activated in an attempt to maintain cardiac output and peripheral perfusion. The adaptive responses are the results of a complex interplay between haemodynamic, myocardial, and neurohormonal mechanisms (Parmley, 1985). Compensatory responses of the cardiovascular system may involve factors that act locally at the site of synthesis. Cardiac myocytes (Suzuki *et al.,* 1993), as well as vascular endothelial cells (Yanagisawa *et al.,* 1988) produce ET-1. Plasma levels of ET-1 and its precursor big ET-1 increase post-MI and are important predictors of outcome, with levels correlating positively with disease severity during development of chronic heart failure (CHF; Rodeheffer *et al.,* 1992; Omland *et al.,* 1994; Wei *et al.,* 1994).

Endogenous ET-1 contributes to maintenance of vascular tone in healthy men (Haynes *et al.,* 1994). Kiowski *et al.* (1995) showed that treatment of CHF patients with the non-selective ET_A/ET_B receptor antagonist bosentan markedly improved haemodynamic parameters and increased cardiac index. Acute administration of ET receptor antagonists was also found to exert favourable haemodynamic effects in rat (Teerlink *et al.,* 1994) and dog (Shimoyama *et al.,* 1996) models of heart failure.

A number of studies have investigated the myocardial ET system in human heart failure. Giaid *et al.* (1995) reported increased ET-1 immunoreactivity and evidence of preproET-1 mRNA in cardiomyocytes and endothelial cells in endocardial biopsy samples from transplanted hearts. Furthermore, ET-1 mRNA expression and immunoreactive ET-1 have been demonstrated in endothelial cells of human cardiac tissue from patients with ischaemic heart disease and idiopathic dilated cardiomyopathy (Plumpton *et al.,* 1996).

In animal studies using rat models of CHF secondary to MI, markedly increased tissue levels of ET-1 peptide, preproET-1, ET_A receptor and ET_B receptor mRNA have been reported in the failing hearts of rats at 3 (Sakai *et al.,* 1996a) and 12 (Sakai *et al.,* 1996b) weeks post-MI compared to respective sham-op rat hearts.

Furthermore, addition of the ET_A selective receptor antagonist BQ-123 10 days post-MI for 12 weeks greatly improved survival, ameliorated LV dysfunction, and prevented ventricular remodelling, indicating that an upregulated ET system may contribute to progression of CHF (Sakai *et al.,* 1996b).

Following MI, the infarcted region of the myocardium undergoes a process of repair involving formation of a collagenous scar (as shown in Chapter 3) that is followed by remodelling of the non-infarcted LV involving hypertrophy and dilatation (Pfeffer & Braunwald, 1990). *In vitro* studies have indicated a role for ET-1 in stimulation of cardiomyocyte hypertrophy (Shubeita *et al.,* 1990; Goto & Warner, 1995) and upregulation of the ET-1 system in the heart may contribute to excessive hypertrophy of the myocardium during CHF. Although the above studies suggest an involvement of the ET-1 system in both myocardial remodelling and hypertrophy post-MI, the specific cellular localisation of preproET-1 and ET receptor expression in areas undergoing hypertrophy, or within the developed scar, later post-MI have not been investigated *in vivo*.

In Chapters 3 and 4, I investigated the myocardial ET-1 system early post-MI with particular reference to scar formation. The aims of this chapter were to further investigate progressive changes in expression of preproET-1, ET_A receptor and ET_B receptor mRNA in areas of the myocardium undergoing repair and remodelling later post-MI. Regions of hypertrophy within the myocardium were identified by distribution of β-myosin heavy chain (MHC), a molecular marker associated with hypertrophy (Michel *et al.,* 1995). Association of ET receptors with the growth factor cytokine TGF-β_1, and the signalling transcription factor ERK, was also investigated, the latter being known to be activated by ET-1 and involved in transcriptional regulation of proto-oncogenes that modulate cell growth and proliferation (Quian *et al.,* 1991; Davis, 1995). In addition, changes in the ET system were investigated within the scar to identify if ET-1 has a role in scar development later post-MI.

5.2 Methods

All procedures were carried out as described in Chapter 2. Rats were investigated twice post-MI, at 5 and 12 weeks, each with respective sham-operated groups. The developed scar was characterised histologically with respect to collagen deposition using van Gieson's stain. Immunohistochemistry was performed using antibodies to GSL I, TGF-β_1, ET-1, ET_B receptor, β-MHC and the extracellular regulated kinase ERK. Expression of preproET-1, ET_A receptor and ET_B receptor mRNA was investigated by *in situ* hybridisation.

5.2.1 Coronary artery ligation rat model

MI was induced by ligation of the left anterior descending coronary artery in male Wistar rats (n=20) as described in Section 2.1. Upon recovery from surgery, rats were housed in groups of 2 and allowed access to normal rat chow and water ad libitum for the duration of the study. All surgical procedures were carried out by Dr. Gillian Gray.

5.2.2 Plasma collection and tissue sampling

Either 5 or 12 weeks after CAL or sham operation, rats were anaesthetised (60mg/kg Na pentobarbital i.p.) and then exsanguinated via the carotid artery where an ~5ml blood sample was collected into a 10ml syringe pre-rinsed with heparin. The blood was aliquoted into pre-chilled test tubes containing 50µl of 10mmol/l final concentration EDTA and immediately centrifuged (2000x*g*, 4°C, 20mins). Plasma was aliquoted off each sample and stored at -70°C for future plasma ET-1 and big ET-1 analysis as described in section 2.2.2.

The heart and lungs were excised, rinsed in ice-cooled physiological saline, then individually weighed. The heart was then bisected longitudinally from apex to base so that each half consisted of both right and left ventricles; a section of the lung was

also separated before all samples were placed in 10% neutral buffered formalin solution for 24hrs fixation prior to further processing and wax embedding. Within the 5 and 12 week post-CAL groups, infarct size as a percentage of the left ventricular wall was measured from 3μM heart sections stained with van Gieson's collagen stain and the nucleus stained with Celestine Blue as described in section 2.3.1.

5.2.3 General staining and immunohistochemistry

3μm wax embedded heart sections were treated with van Gieson's collagen stain highlighting collagen as described in section 2.3.3. Immunohistochemistry was also performed on 3μm heart sections using antibodies to GSL, ET-1 and TGF-β_1 using the alkaline phosphatase detection method, and antibodies to ET_B receptors, β-MHC and ERK using the peroxidase detection method as described in sections 2.4.2 and 2.4.3.

5.2.4 *In situ* hybridisation

In situ hybridisation was performed on 3μm wax embedded heart sections using probes to preproET-1, ET_A and ET_B receptor mRNA as described in sections 2.5.3-2.5.5. PreproET-1, ET_A receptor and ET_B receptor mRNA were semi-quantified in heart sections using a computerised image analysis system (Zeiss Kontron 300, Image Associates, UK) as described in section 2.6.1. *In situ* procedures were developed and carried out by Dr. Pauline McEwan.

5.2.5 Statistical analysis

All data was displayed as mean±SEM. *In situ* hybridisation data were analysed between groups by one-way ANOVA and significance was established within groups using a Student's *t*-test. Unpaired observations between organ weights and plasma levels from 5 and 12-week MI and respective sham groups were assessed by Student's *t*-test. A *P* value of <0.05 was considered significant.

5.3 Results

5.3.1 Effects of coronary artery ligation

Survival rate during the study period was 70%. Death was limited to the first 24-hour period after surgery. *Table 5.1* below summarises organ weights, LV infarct sizes, and plasma ET-1 and big ET-1 values from the relevant groups.

Table 5.1 Table of organ weights, infarct sizes and plasma levels.

	5 Week Sham-op	**5 Week post-MI**	**12 Week Sham-op**	**12 Week post-MI**
Heart Weight (g/kg Body Weight)	2.95±0.11 (n=7)	*3.48±0.16 (n=7)	3.12±0.14 (n=7)	3.17±0.70 (n=5)
Lung Weight (g/kg Body Weight)	3.62±0.15 (n=7)	5.13±0.91 (n=7)	3.6±0.17 (n=7)	4.40±0.09 (n=5)
Infarct Size (as % of LV free wall)	No Infarct	43.6±8.4 (n=6)	No Infarct	49.0±11.2 (n=4)
Plasma ET-1 (pg/ml)	3.1±0.8 (n=3)	3.2±1.2 (n=3)	5.0±0.8 (n=5)	5.9±2.1 (n=3)
Plasma big ET-1 (pg/ml)	24.8±3.0 (n=3)	23.7±2.8 (n=3)	26.4±5.2 (n=5)	38.1±14.3 (n=3)

Key: *P<0.05 versus respective sham-op group.

Organ weights were not measured for 2 of the 12-week post-MI rats. Furthermore, due to difficulties encountered in measuring plasma ET-1 and big ET-1, it was not possible to obtain values for all of the rats. Heart weights were significantly increased at 5 weeks, but not 12 weeks, post-MI compared to the sham-op group. Lung weights tended to be increased at both time points in the post-MI groups, though no significant difference was observed. From the plasma values gained, levels of big ET-1 tended to be higher in the post-MI group at 12 weeks compared to the respective sham-op group, though plasma ET-1 levels remained similar between groups.

5.3.2 Distribution of preproET-1, ET_A and ET_B receptor mRNA.

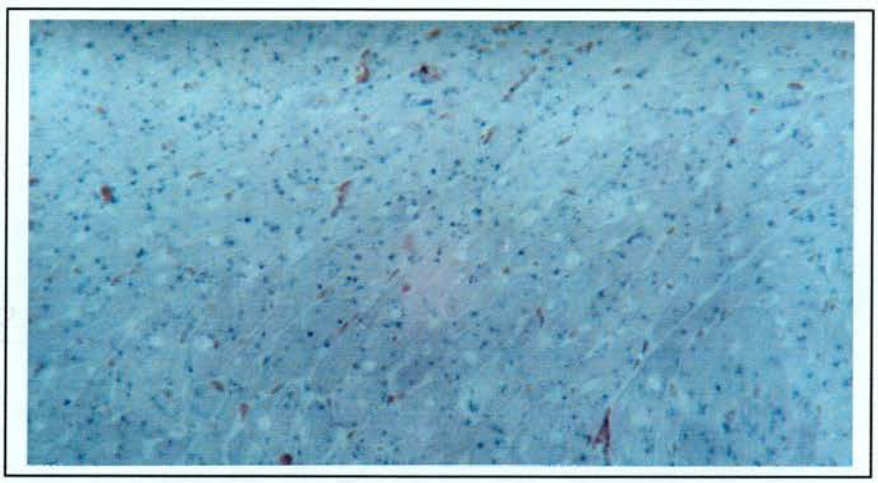
Within the sham-op hearts, preproET-1 mRNA was expressed abundantly throughout all chambers, being located in cardiomyocytes in both atria and ventricles (*Figure 5.1 (a)*). Within the 5-week and 12-week post-MI hearts, preproET-1 mRNA was again expressed abundantly throughout atria, RV, septum and non-infarcted LV (*Figure 5.1 (b, c)*). Distribution of preproET-1 mRNA expression was similar to that of ET-1 immunoreactivity (*Figure 5.8 (a, b, c)*). At 5 and 12 weeks post-MI within the infarct, preproET-1 mRNA was present only in remaining cardiomyocytes, with areas of collagen deposition showing little expression (*Figure 5.1 (d)*). Levels within the infarct were therefore significantly decreased ($P<0.001$, *Figure 5.2 (a, b)*) compared to respective LV areas in myocardium from sham-op rat hearts. However, expression in the RV and non-infarcted LV away from the scar remained unchanged (*Figure 5.2 (a, b)*). For preproET-1 and subsequent expression studies using ET_A and ET_B receptor antisense, treatment of consecutive sections with appropriate sense control probes showed little evidence of non-specific binding or background activity (*Figure 5.1 (e), Figure 5.3 (c)*).

In sham-op myocardium, ET_A receptor mRNA was localised throughout the heart in atria, RV and LV (*Figure 5.3 (a)*) and within the vascular media of intramyocardial blood vessels (not shown). A similar distribution of ET_A receptor mRNA was found in myocardium from 5 (*Figure 5.3 (b)*) and 12-week post-MI rat hearts except within the infarct where ET_A mRNA levels were significantly lower ($P<0.01$ at 5 weeks post-MI and $P<0.001$ at 12 weeks post-MI, *Figure 5.4 (a, b)*), being restricted to the few remaining cardiomyocytes with no evidence of ET_A mRNA in deposited collagen within the scar (not shown). There was no difference in ET_A expression within the RV, septum or non-infarcted LV of hearts from the 5 or 12-week post-MI groups compared to sham-op controls (*Figure 5.4*).

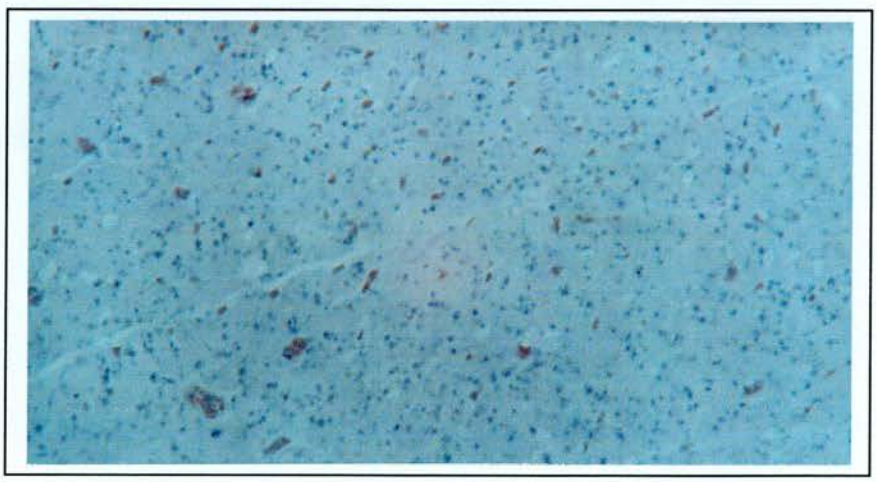
ET_B receptors were expressed at low levels throughout the hearts of sham-op rats and could be observed as small clusters in cardiomyocytes in both RV and LV (*Figure 5.5 (a)*). ET_B receptor mRNA was similarly distributed within cardiomyocytes in the

Figure 5.1 PreproET-1 mRNA in situ hybridisation images from sections representative of hearts from the 5 and 12-week groups. PreproET-1 mRNA expression in (a) the LV of a sham-op heart; and (b) in the non-infarcted LV of a 5-week post-MI, and (c) 12-week post-MI heart (arrows highlighting signal in cardiomyocytes); (d) the infarct border of a 5-week post MI heart showing lack of mRNA expression in collagen fibrils (arrows); and (e) a sense slide showing an area within the RV of a 5-week sham-op heart (arrows indicate red blood cells in capillary vessels, all images x400 magnification).

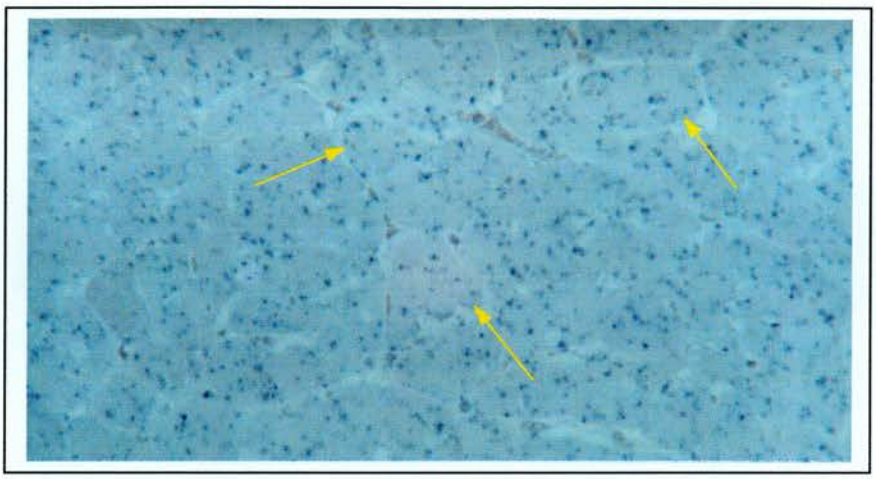
(a)



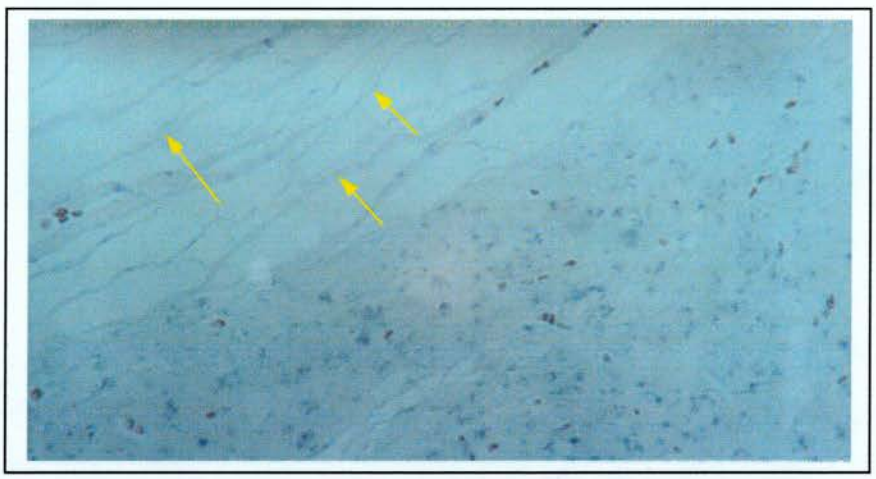
(b)



(c)



(d)



(e)

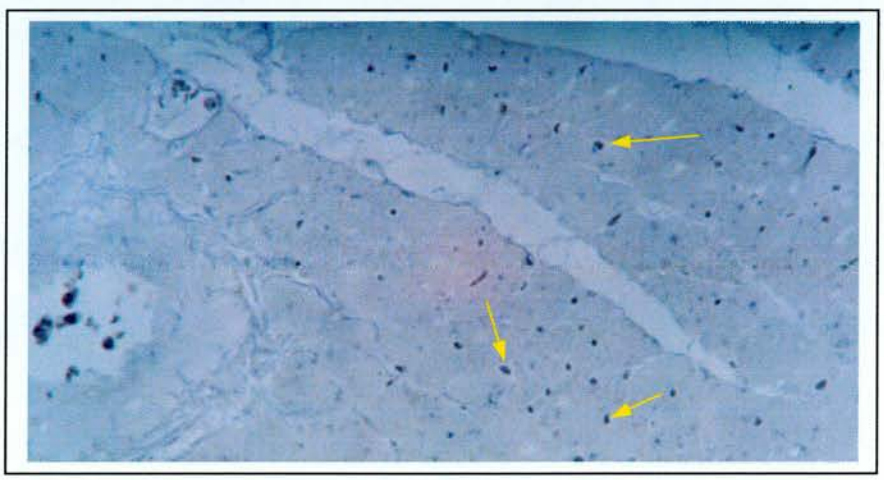


*Figure 5.2. PreproET-1 mRNA expression in the right ventricle (RV), area of infarction or sham-operation (OP/INF), and left ventricle (LV) of rat hearts at (a) 5 and (b) 12 weeks post-MI or sham-operation (n=7). **P<0.01 versus respective sham-op group.*

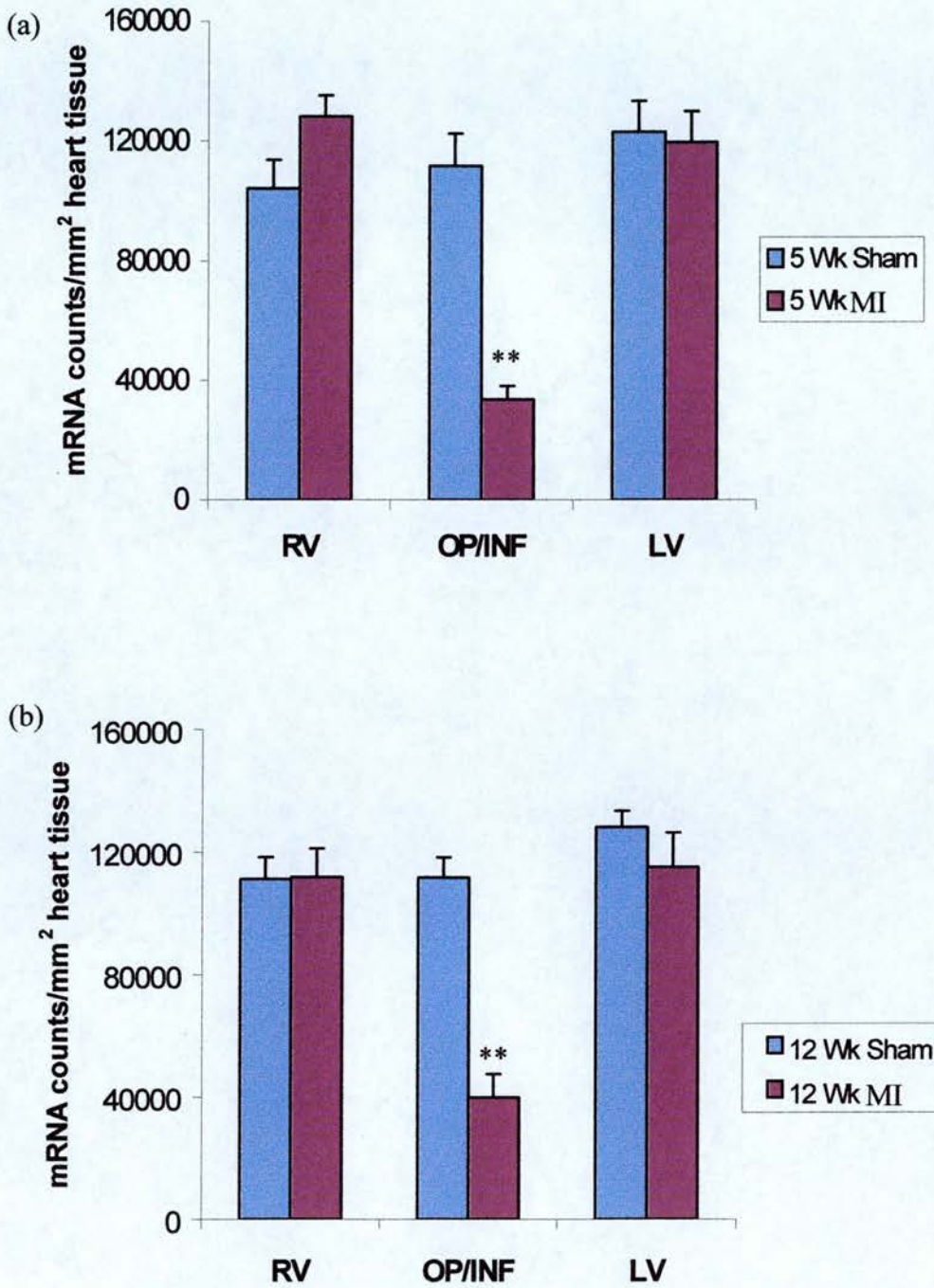
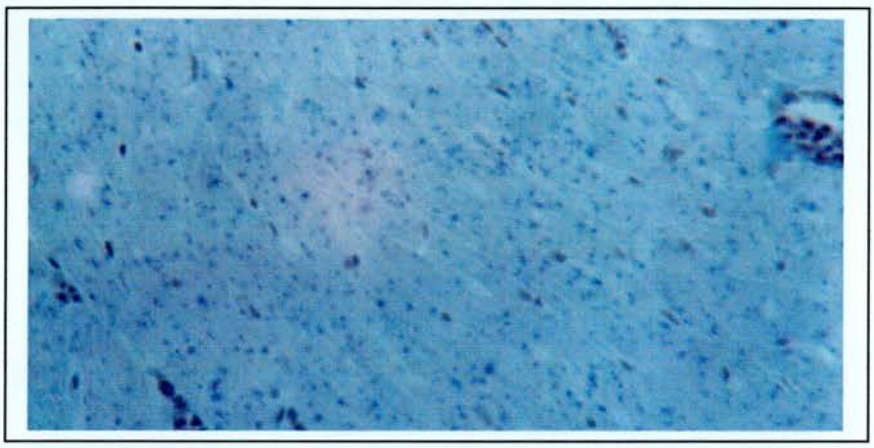
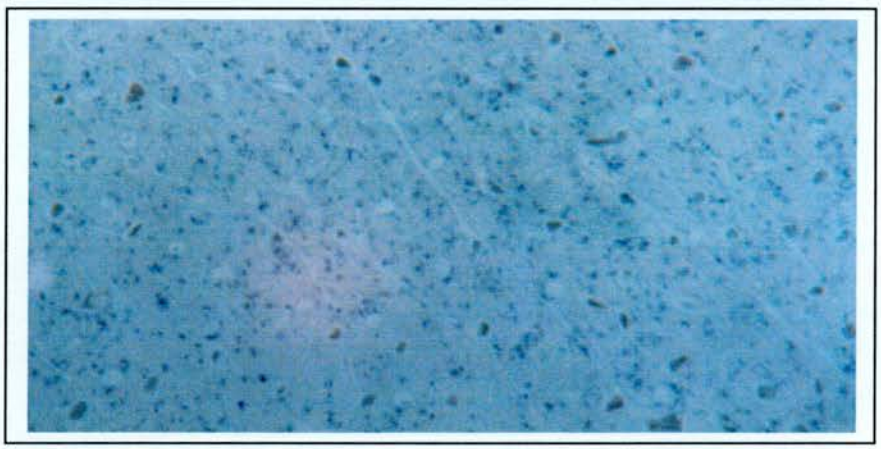


Figure 5.3 In situ hybridisation images of ET_A receptor mRNA from heart sections representative of the 5-week groups. ET_A receptor mRNA expression in (a) the LV of a 5-week sham heart (x400 magnification); (b) the non-infarcted LV of a 5-week post-MI heart (x400 magnification), and (c) a sense section showing no ET_A receptor mRNA expression (x400 magnification, arrows indicate red blood cells within capillary vessels).

(a)



(b)



(c)

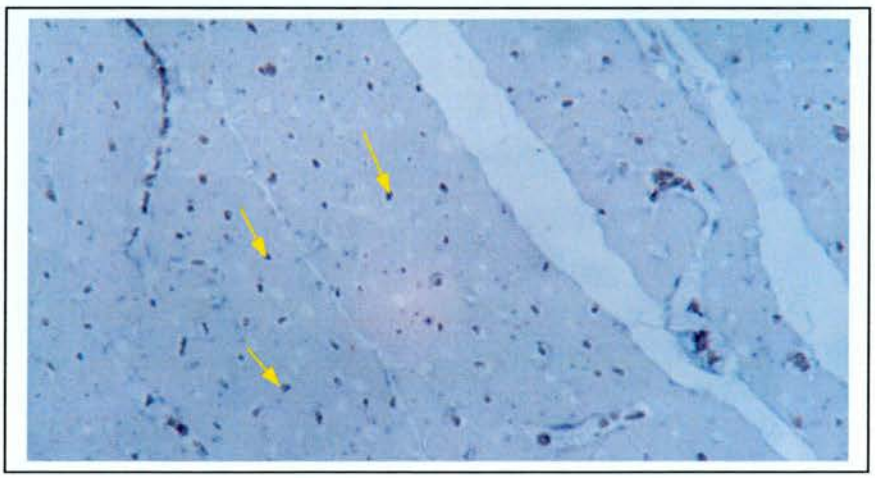


*Figure 5.4. ET_A receptor mRNA expression in the right ventricle (RV), area of infarction or sham-operation (OP/INF), and left ventricle (LV) of rat hearts at (a) 5 and (b) 12 weeks post-MI or sham-operation (n=7). **P<0.01 ***P<0.001 versus respective sham-op group.*

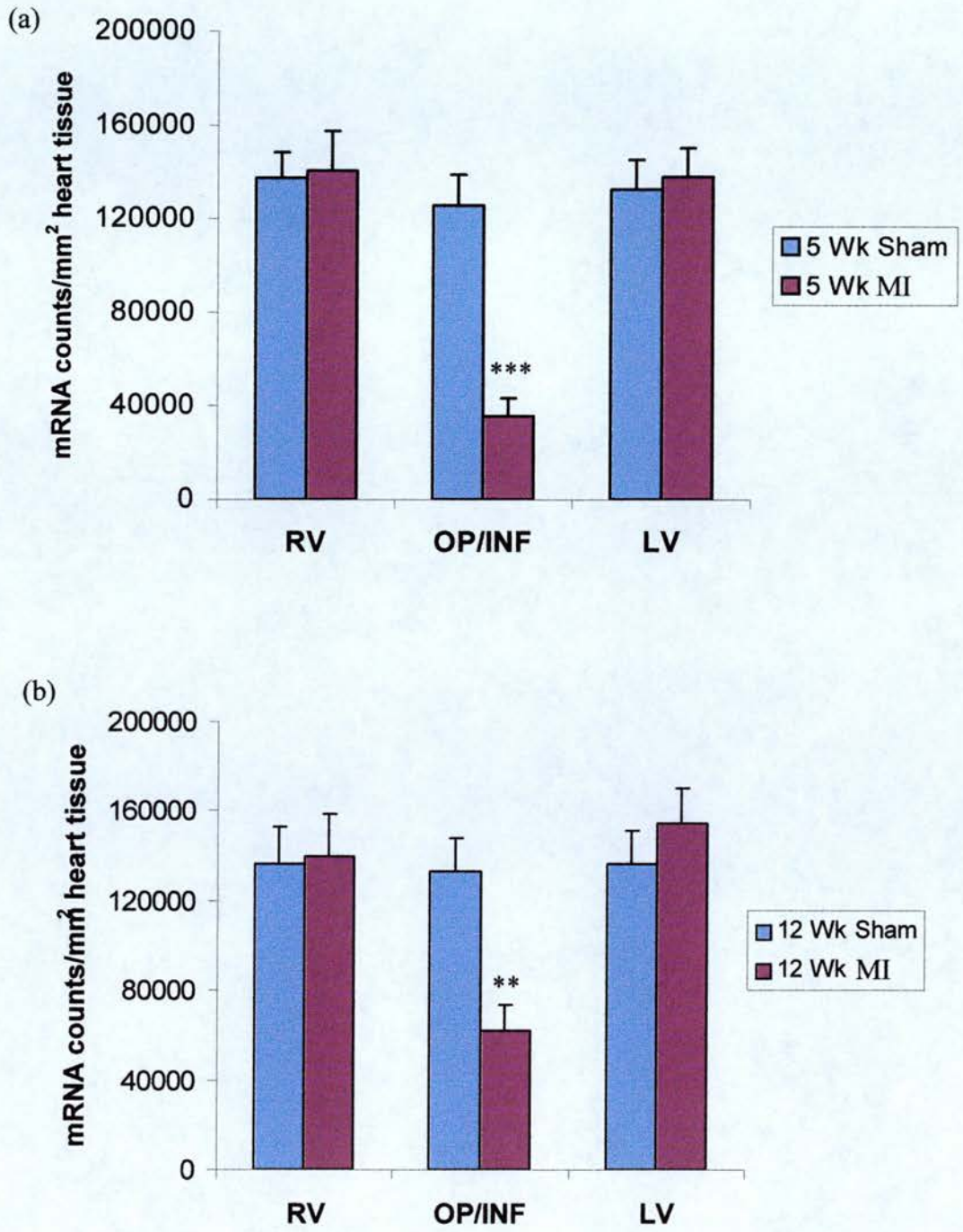
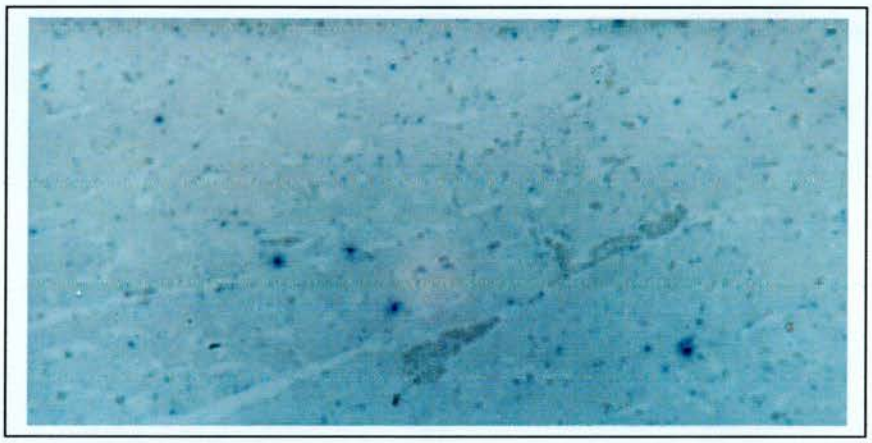
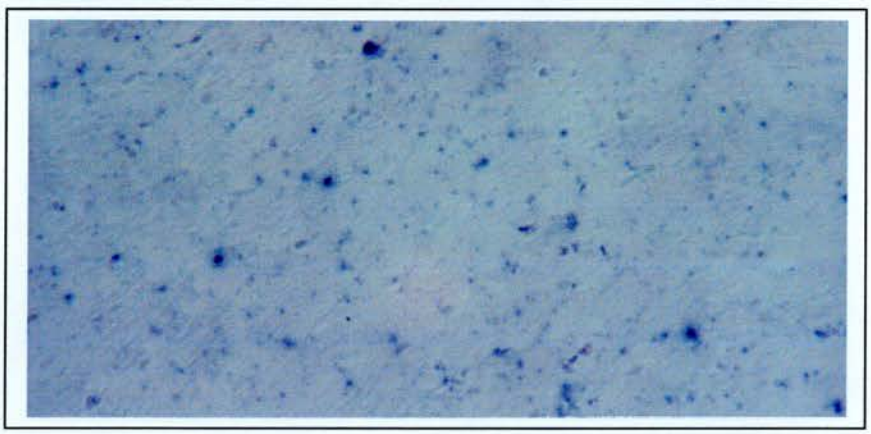


Figure 5.5 In situ hybridisation images of ET_B receptor mRNA from heart sections representative of 5 and 12-week groups. ET_B receptor mRNA expression in (a) the LV of a 5-week sham heart; (b) the non-infarcted LV of a 5-week post-MI heart and (c) 12-week post-MI heart (all images x400 magnification, arrows indicate expression in cardiomyocytes).

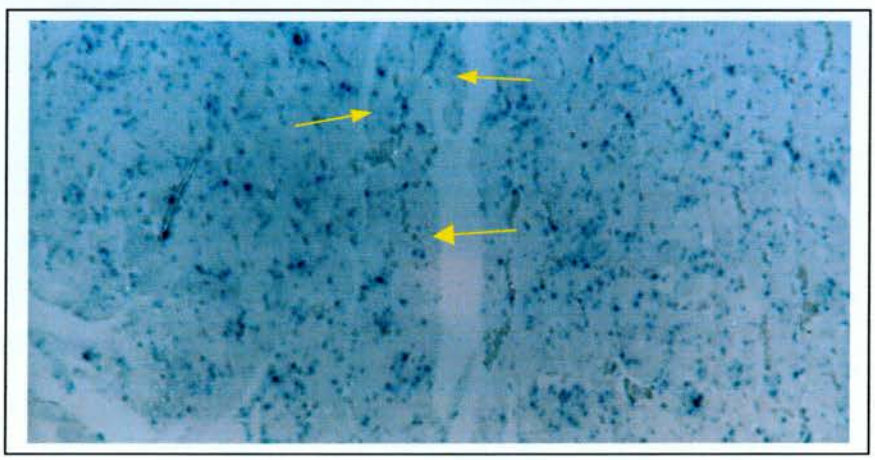
(a)



(b)



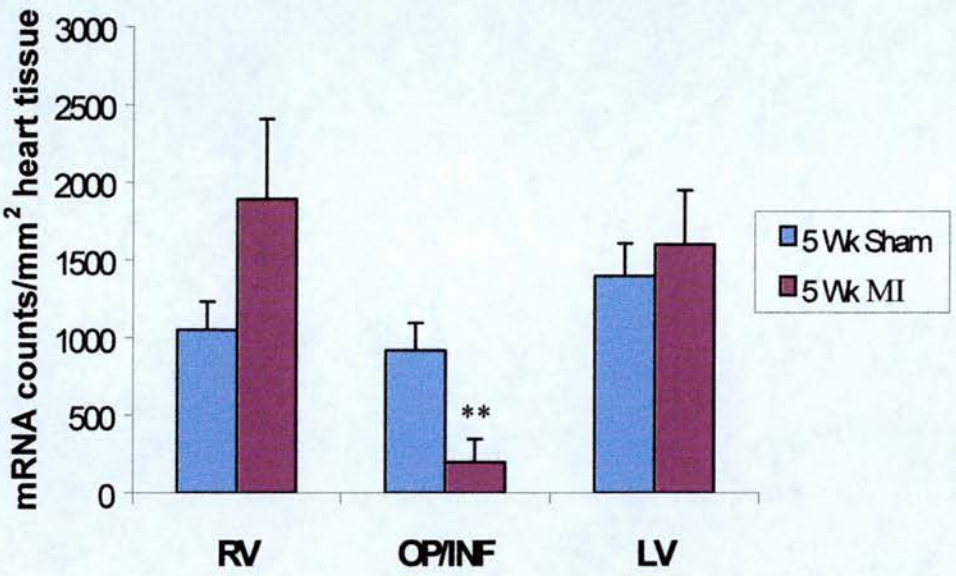
(c)



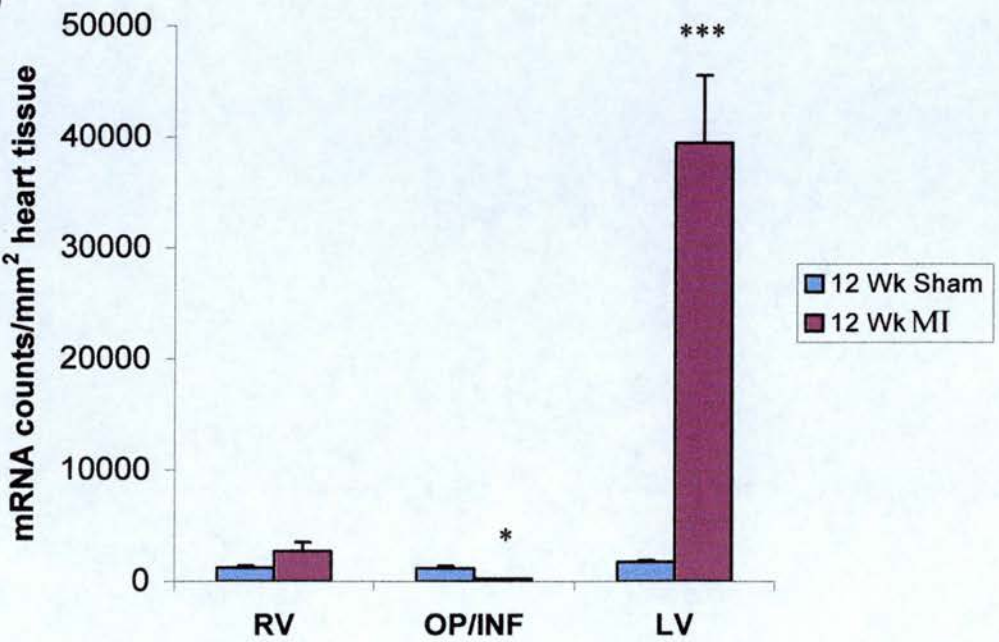
RV, LV and septum, whereas ET_B receptor protein was confined to myoendothelial and vascular endothelial cells in sham-op hearts (*Figure 5.9 (a)*). ET_B receptor mRNA levels were significantly lower within the infarct at 5 ($P<0.01$, *Figure 5.6 (a)*) and 12 ($P<0.05$, *Figure 5.6 (b)*) weeks post-MI, being restricted to remaining cardiomyocytes (not shown). At 5 weeks post-MI, there were no changes in ET_B mRNA expression in the non-infarcted LV, RV or septum (*Figure 5.6 (a)*) compared to sham controls. However, at 12 weeks post-MI in the non-infarcted LV, ET_B mRNA expression increased by approximately 20-fold compared with sham-op controls ($P<0.001$, *Figure 5.5 (b)*, *Figure 5.6 (b)*). The increase in ET_B receptor mRNA in the non-infarcted LV at the later time point coincided with the specific increase in ET_B receptor protein also observed only at this time point. Low levels of ET_B receptor mRNA expression were found in the RV and septum, similar to those observed in the myocardium of hearts from sham-op group rats (*Figure 5.6 (b)*).

*Figure 5.6. ET_B receptor mRNA expression in the right ventricle (RV), area of infarction or sham-operation (OP/INF), and left ventricle (LV) of rat hearts at (a) 5 and (b) 12 weeks post-MI or sham-operation (n=7). *P<0.05, **P<0.01 ***P<0.001 versus respective sham-op group.*

(a)



(b)



5.3.3 Histological and immunohistochemical characterisation.

A thinned, defined, scar was present in 6 of the 5-week post-MI and 4 of the 12-week post-MI rat hearts. For those hearts not showing a thinned scar, an area of myocardial damage was evident below the ligation. Scars consisted predominantly of mature cross-linked collagen fibrils with a few remaining cardiomyocytes (*Figure 5.7 (a, b)*). There was evidence of a few microvessels present within the scar with larger, more established, vessels also observed throughout the scar region (*Figure 5.7 (c)*).

ET-1 immunoreactivity at both 5 and 12 weeks was diffuse throughout the non-infarcted LV (*Figure 5.7 (d), Figure 5.8 (a)*), with distribution and intensity of staining similar to that observed in the RV and septum of post-MI rats and throughout the myocardium of sham-op rat hearts (not shown). Within the infarct, ET-1 staining was sparse at both time points (*Figure 5.7 (d), Figure 5.8 (a)*), being confined to remaining cardiomyocytes (*Figure 5.7 (e), Figure 5.8 (c)* and a few inflammatory cells and fibroblasts (not shown).

To verify distribution of immunoreactive staining using the ET-1 antibody from Biodesign, UK, another ET-1 antibody was used (a gift from Dr. A. Davenport), this antibody being produced in-house and directed towards mature ET-1 peptide. The antibody from Dr. Davenport's laboratory demonstrated ET-1 immunoreactivity again located in cardiomyocytes throughout the non-infarcted LV, RV and septum, with sparse staining in the infarct region of heart sections from the 12-week post-MI group (*Figure 5.8 (b)*). Distribution of immunoreactivity was identical to that found with the antibody used from Biodesign, UK (*Figure 5.8 (a)*).

ET_B receptor protein immunoreactivity was confined to myoendothelial and vascular endothelial cells in sham-op hearts (*Figure 5.9 (a)*). At 5 and 12 weeks post-MI, ET_B receptor immunoreactivity was scarce within the infarct (not shown). In the non-infarcted LV at 12 weeks but not 5 weeks post-MI, ET_B receptor immunoreactivity was increased specifically in the area adjacent to the infarct, and could also be

Figure 5.7 Images from sections representative of 5-week post-MI group hearts. (a) van Gieson's staining of the non-infarcted LV and infarct (x50 magnification), and (b, ★) a magnified image from within the infarct of mature collagen fibrils (x200 magnification); (c, †) remaining microvessels within the infarct highlighted with GSL I (arrows, x200 magnification); (d) a parallel section to (a) showing ET-1 staining in the non-infarcted LV with sparse staining in the infarct (x50 magnification) and (e) a magnified area of the infarct showing ET-1 immunoreactivity in remaining cardiomyocytes (x100 magnification).

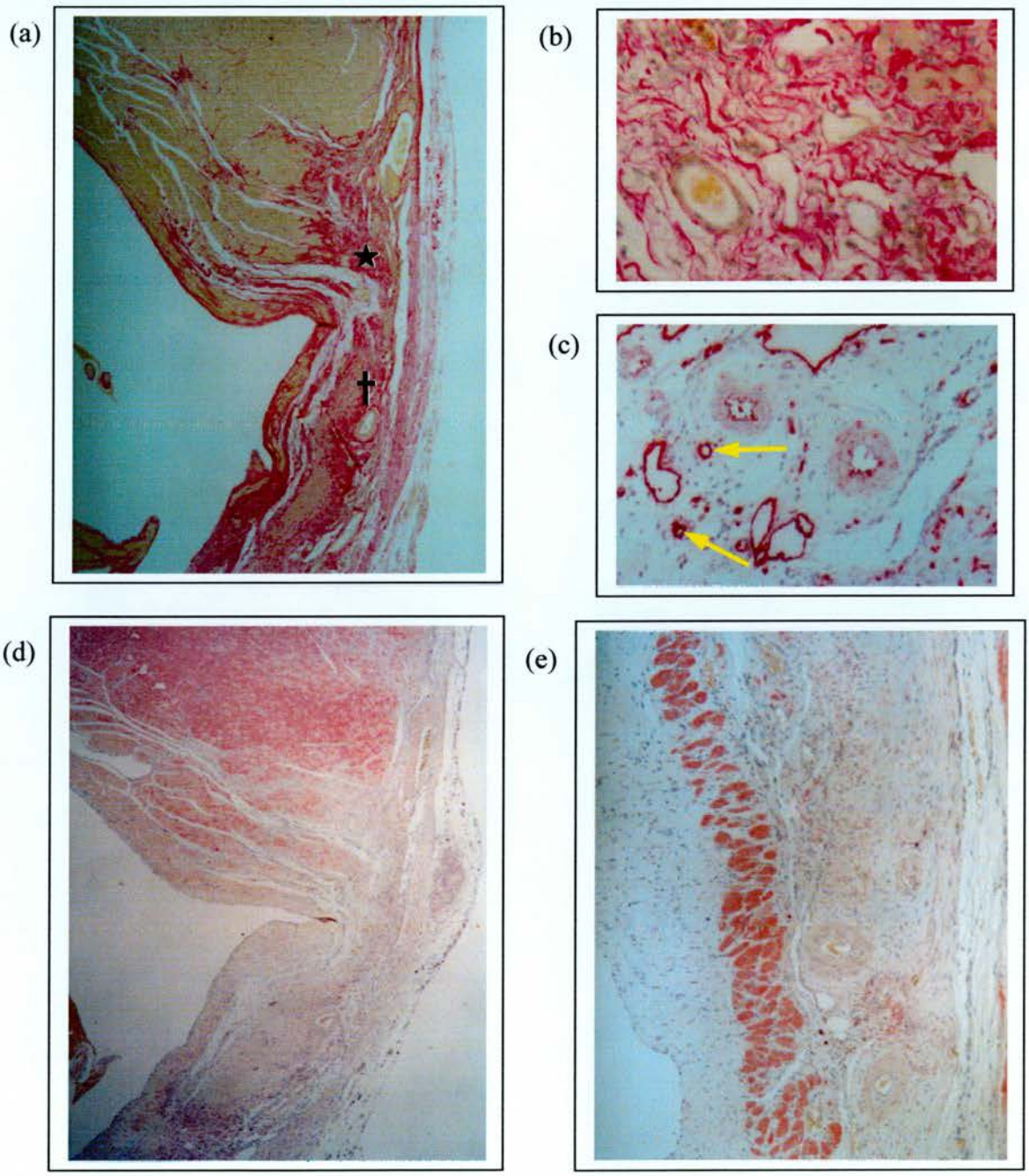
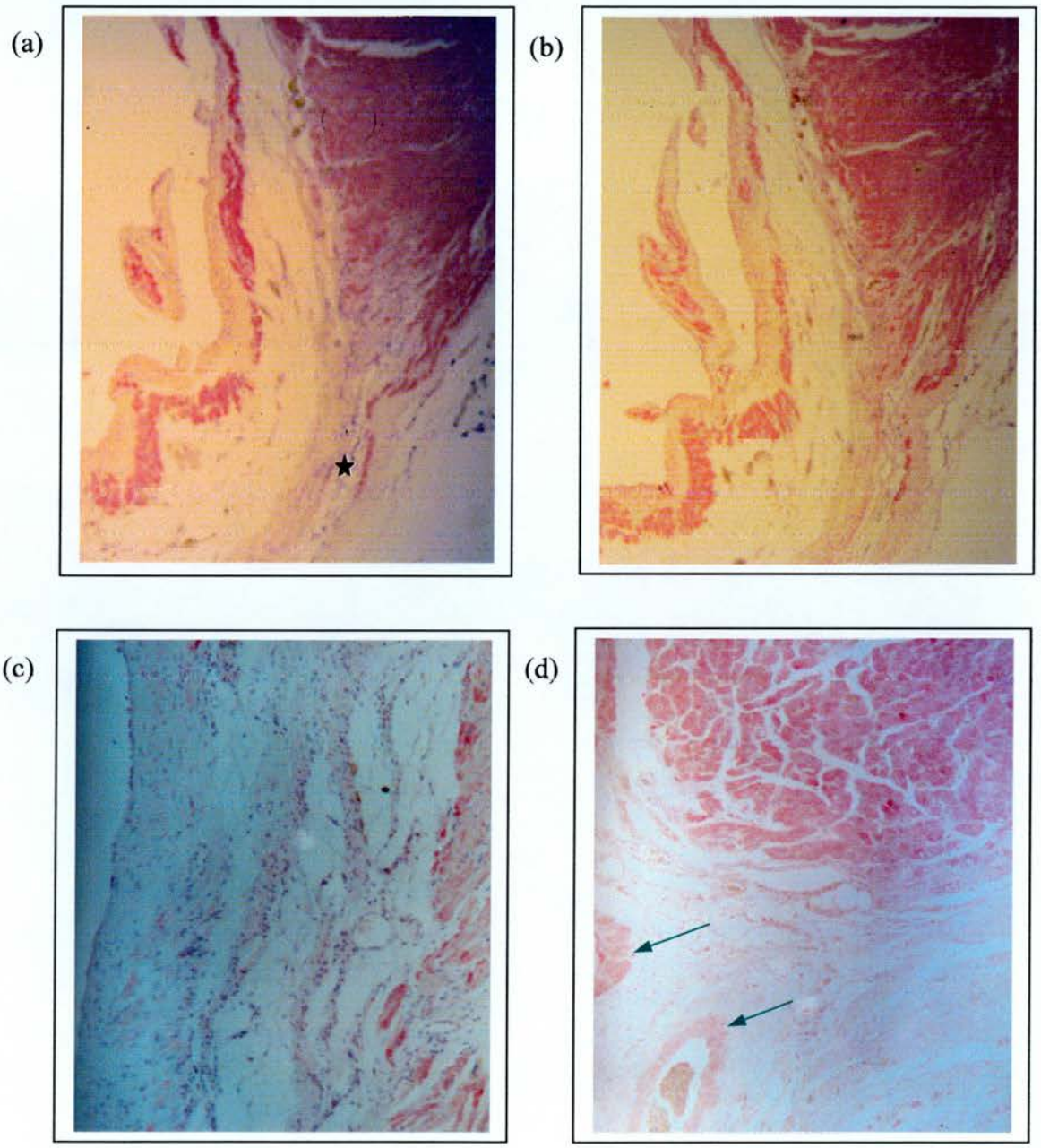


Figure 5.8 Images from sections representative of the 12-week post-MI group hearts. ET-1 immunoreactivity in (a) the non-infarcted LV border with sparse staining in the infarct, using an ET-1 specific antibody from Biodesign, UK, and (b) a parallel section showing identical ET-1 staining using an antibody directed toward mature ET-1 peptide (a gift from A. Davenport, both images x50 magnification); (c, ★) a magnified area from within the infarct of (a), ET-1 is restricted to remaining cardiomyocytes (x200 magnification); and (d) TGF-β_1 staining in the non-infarcted LV and remaining cardiomyocytes (arrows) in the infarct (x200 magnification).



located in cardiomyocytes (*Figure 5.9 (b)*). ET_B receptor protein staining in the RV and septum was similar to that observed throughout hearts in the 5-week post-MI and sham-op groups.

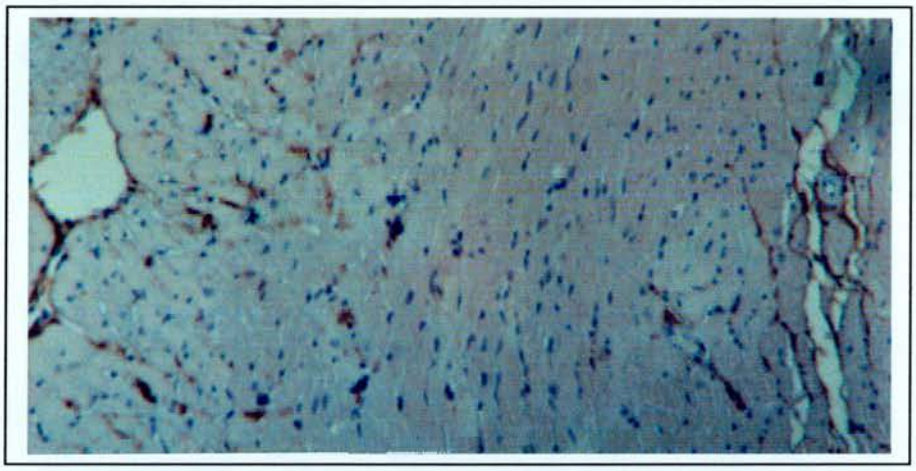
TGF-β_1 immunoreactivity was sparse within the infarct at 5 and 12 (*Figure 5.8 (d)*) weeks post-MI, being located in only a few vascular endothelial cells and fibroblasts (not shown). However, increased staining was present in cardiomyocytes close to, and adjacent to, the infarct border (*Figure 5.8 (d)*). Distribution and intensity of staining in the RV and septum at both times post-MI was similar to that found in the myocardium from respective sham-op group rats (not shown).

β-MHC immunoreactivity was sparse in the RV and LV of hearts from both 5 and 12-week (*Figure 5.10 (a)*) sham-op groups. At 5 & 12 weeks post-MI however, areas of intense staining within cardiomyocytes were evident in the non-infarcted LV (*Figure 5.10 (b, c)*). Remaining cardiomyocytes within the area of infarction were also intensely stained, but immunoreactivity was greatest in the non-infarcted LV.

ERK was detected in myoendothelial cells, cardiomyocytes (*Figure 5.11 (a)*) and vascular smooth muscle cells of intramyocardial blood vessels in sham-op rat hearts. Although ERK was present in both RV and LV, the LV of hearts from sham-op and post-MI rats showed higher levels of expression than the RV (not shown). At 5 weeks post-MI, ERK was present in intact cardiomyocytes and in the vascular smooth muscle layer of blood vessels within the scar region of the infarct (not shown). ERK was also present in the non-infarcted LV (*Figure 5.11 (b)*), though showed a similar level of immunoreactivity to that observed in the 5-week sham controls. At 12 weeks post-MI however, the highest intensity of ERK staining was detected in the non-infarcted LV (*Figure 5.11 (c)*), with staining greater than that observed at 5 weeks.

Figure 5.9 Images of ET_B immunoreactivity (brown) in sections representative of 12-week group hearts. Nuclei are stained purple with haematoxylin. ET_B staining within (a) the LV of a 12-week sham heart, and (b) the non-infarcted LV of a 12-week post-MI heart (both images x200 magnification).

(a)



(b)

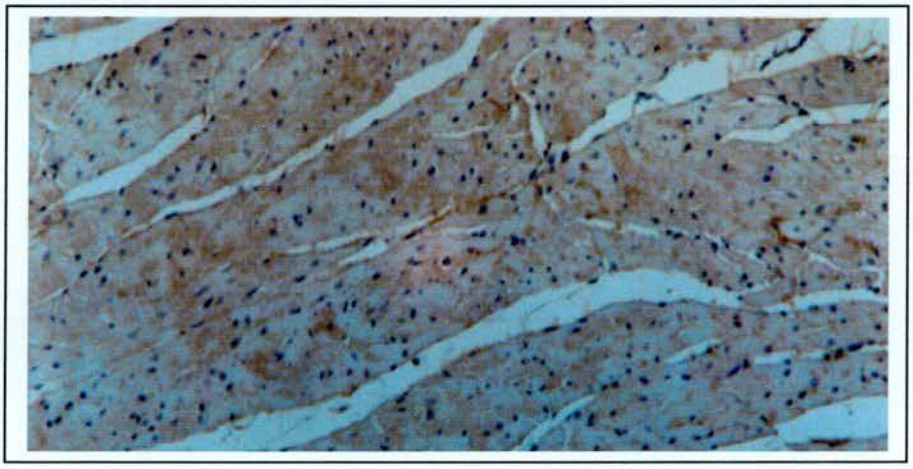
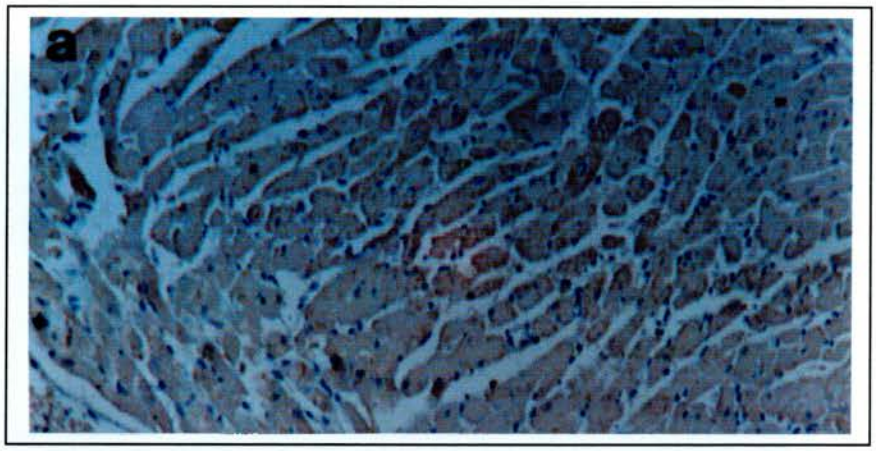
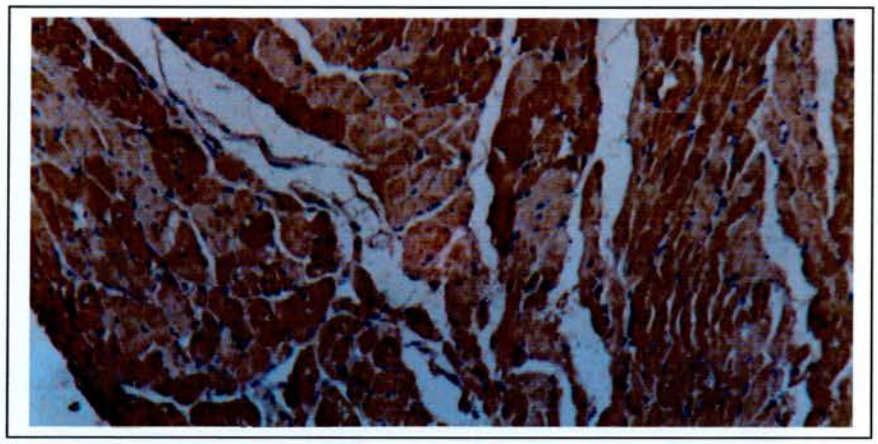


Figure 5.10 Images showing β-MHC immunoreactivity (brown) in sections from 5 and 12-week group hearts. Nuclei are stained purple with haematoxylin. β-MHC staining within (a) the LV of a 12-week sham heart; (b) the non-infarcted LV of a 5-week heart, and (c) 12-week post-MI heart (all images x400 magnification).

(a)



(b)



(c)

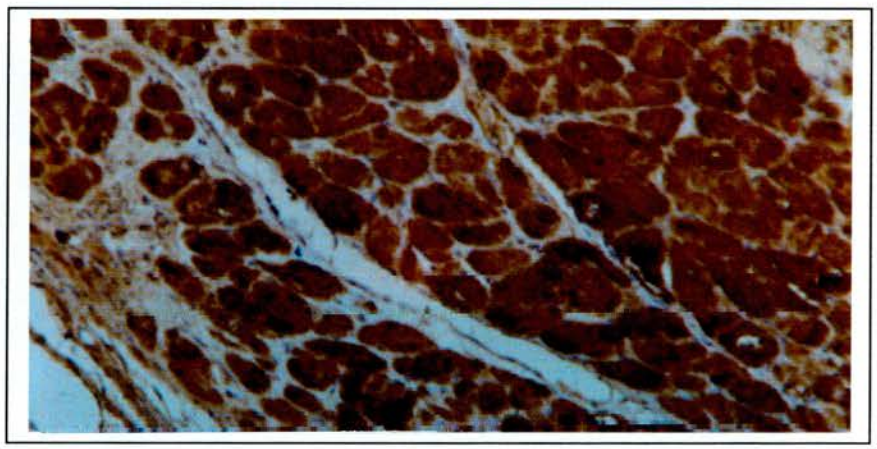
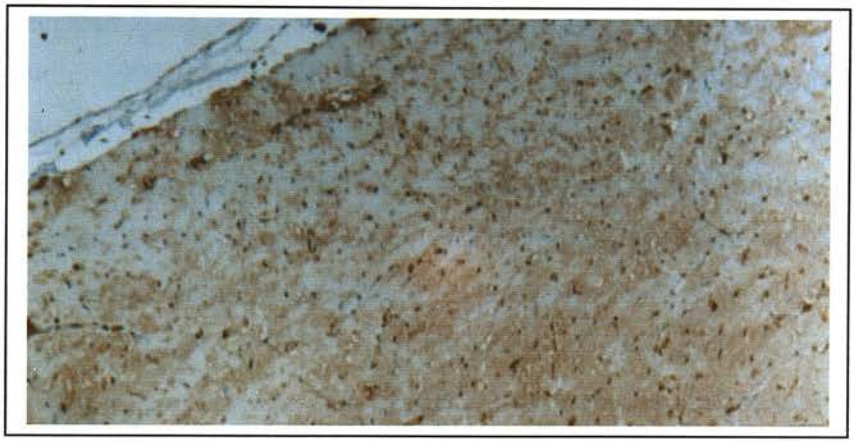
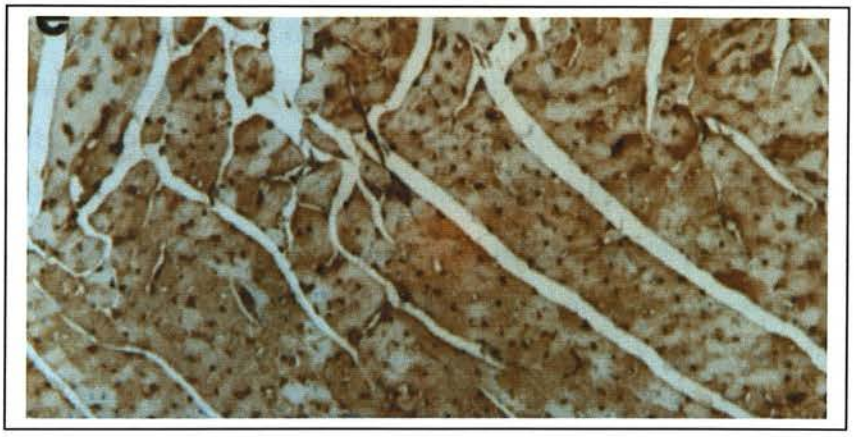


Figure 5.11 Images of ERK immunoreactivity (brown) from sections representative of 5 and 12-week group hearts. Nuclei are stained purple with haematoxylin. ERK staining within (a) the LV of a heart from the 5-week sham-op group; and (b) the non-infarcted LV of hearts from the 5-week post-MI, and (c) 12-week post-MI groups (all images x400 magnification).

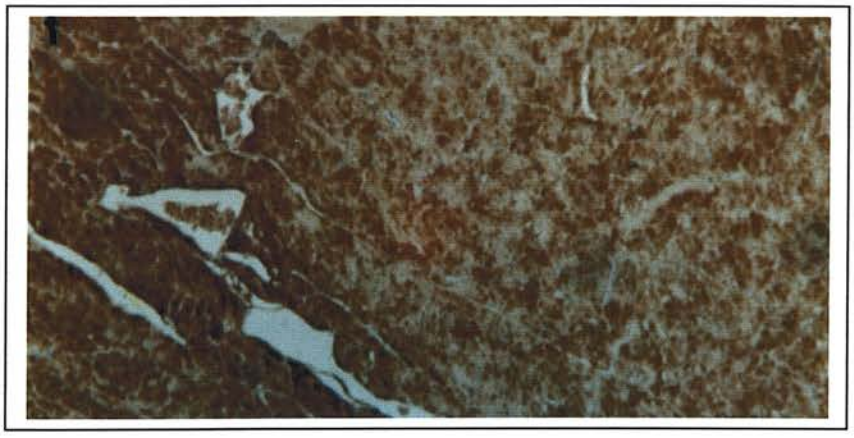
(a)



(b)



(c)



5.4 Discussion

Coronary artery ligation resulted in a myocardial infarction (MI) with infarct sizes 40-50% of the LV free wall and a mortality of 30% that was limited to the 24-hour period after surgery. This rat model, therefore, resembled others demonstrating moderate infarction (Fraccarollo *et al.,* 1997; Mulder *et al.,* 1997; Mulder *et al.,* 1998) rather than those with large infarctions and higher mortality (Sakai *et al.,* 1996a; 1996b; 1998; Kobayashi *et al.,* 1999). Heart weight was significantly increased at 5 weeks, but not 12 weeks, post-MI compared to sham-op controls. The lack of a significant increase observed at 12 weeks may have been due to a lower number of heart weight values in the post-MI group, as heart weight was not recorded for 2 of the animals at the time of experiment.

Plasma ET-1 levels were similar in both post-MI groups compared to their respective sham-op groups. Big ET-1 levels were also similar in the post-MI and sham-op groups at 5 weeks, however there was a tendency for big ET-1 levels to increase at the later time post-MI compared to the respective sham-op group. Due to the small group numbers for plasma values, it is difficult to interpret these data accurately. However, the observed tendency for elevated plasma big ET-1 at 12 weeks post-MI may be associated with a progression of heart failure and an increased synthesis of ET-1. Big ET-1, having a more stable half-life and slower clearance rate from the plasma than ET-1 is thought to correlate more reliably with progression of heart failure than plasma ET-1 itself (Burkhardt *et al.,* 2000). Plasma ET-1 was found to increase only in patients with moderate (NYHA class III) or severe (NYHA class IV) heart failure compared with healthy subjects and individuals with asymptomatic (NYHA class I) or mild (NYHA class II) heart failure (Wei *et al.,* 1994), and in animals with severe CHF (Sakai *et al.,* 1996a).

In sham-op hearts, preproET-1 mRNA and ET-1 peptide immunoreactivity was widespread throughout all 4 chambers of the heart and was associated with cardiomyocytes and the endothelium of coronary blood vessels (McEwan *et al.,* 1998b). ET_A receptor mRNA was also found abundantly throughout the

myocardium, consistent with its role as the major receptor mediating the effects of ET-1 in cardiomyocytes (Miyauchi & Goto, 1999). ET_B receptor mRNA was detected in lesser quantities and was also located in cardiomyocytes throughout the myocardium. The observation of preproET-1 mRNA expression in cardiomyocytes throughout the myocardium correlating with the distribution of ET-1 peptide immunoreactivity, and the presence of ET_A and ET_B receptor mRNA in similar cells, suggests the presence of a local cardiac ET system in the heart with the ability to synthesise, and be stimulated by, ET-1. These findings complement previous studies demonstrating a local ET production in the heart, with ET-1 secreted from the endocardium, myocardium, and the coronary endothelium, thereby acting in a paracrine and autocrine fashion on myocytes (Suzuki *et al.,* 1993; Evans *et al.,* 1994). It further adds to these studies by localising the distribution of preproET-1, ET_A and ET_B receptor mRNA to cardiomyocytes throughout the myocardium. The present study is also in agreement with human studies showing expression of endothelin peptides and mRNA in the heart (Plumpton *et al.,* 1996) and autoradiographic studies showing the existence of ET receptors in human ventricular myocardium (Bax *et al.,* 1993; Molenaar *et al.,* 1993).

At 5 and 12 weeks post-MI, levels of preproET-1 and ET_A receptor mRNA in the RV, septum or non-infarcted LV areas of the heart were similar to that observed in respective areas in sham-op controls. A similar distribution of ET-1 peptide immunoreactivity to that of preproET-1 mRNA was detected in the non-infarcted LV, RV and septum of post-MI rat hearts and to that throughout the myocardium of sham-op rat hearts. At 5 and 12 weeks post-MI, the infarcted region consisted of a thinned, mature collagen-filled scar containing few cardiomyocytes and some remaining inflammatory cells and microvessels. Levels of preproET-1, ET_A receptor and ET_B receptor mRNA within the infarct were significantly reduced at both times compared to a similar area in respective sham-op hearts. mRNA for preproET-1 and ET_B receptors could be detected only in remaining cardiomyocytes, and for ET_A receptors in cardiomyocytes and vascular medial smooth muscle cells. Distribution of ET-1 and TGF-β_1 peptide staining was also reduced compared to that observed previously in the developing scar during the 2 weeks post-MI, with immunoreactivity

being restricted to cardiomyocytes in the peri-infarct area and a few remaining inflammatory cells near to the infarct border. The observation of reduced ET-1 staining in the infarct supports previous findings of reduced ET-1 immunoreactivity within the scar observed at 3 (Kobayashi *et al.,* 1999) and 6 (Øie *et al.,* 1997) weeks post-MI in the rat. Overall, these studies, along with the findings in Chapter 3 and 4, suggest a transient upregulation of ET-1 within the scar with increased levels of immunoreactivity associating with development of a mature scar over the first 2 weeks only.

Previous animal studies investigating the ET-1 system in hearts post-MI have reported varying levels of expression and immunoreactivity, depending on severity of infarct and duration of study post-MI. Kobayashi *et al.* (1999) reported upregulation of preproET-1, ET_A receptor and ET_B receptor mRNA throughout hearts from rats 3 weeks after chronic MI (infarct ~70% of the LV). Other studies by the same group also showed increased preproET-1 mRNA expression and ET-1 peptide immunoreactivity in the LV at 3 (Sakai *et al.,* 1996a) and 12 (Sakai *et al.,* 1996b) weeks after chronic MI in the rat. In a less severe rat model of MI, where infarcts were ~40-50% of the LV, levels of preproET-1 mRNA were found to increase substantially in the infarcted and non-infarcted LV 1 week post-MI, before returning towards baseline at 6 weeks post-MI (Øie *et al.,* 1997, Tønnessen *et al.,* 1998). The similar level of expression of preproET-1 in the non-infarcted LV at both time points post-MI in the present study agrees with findings by Øie and Tønnessen, using rats with similar infarct sizes. This contrasts with the results from Sakai's group who showed upregulation of the ET system in rats with severe infarcts. Taken together, these studies suggest a relationship between the severity of infarction and activation of the myocardial ET system post-MI.

At 12 weeks, but not 5 weeks, post-MI a profound increase in ET_B receptor mRNA expression was observed in the non-infarcted LV only, with levels significantly greater than those observed in the RV or septum of the same hearts, and also than those found throughout 12-week sham-op hearts. The increase in ET_B receptor mRNA expression was associated with an increase in ET_B receptor protein

immunoreactivity, indicating an increase in expression of functional receptors. Gray *et al.* (2000) also recently reported specific upregulation of ET_B receptor immunoreactivity, at 12 weeks but not 5 weeks, within the media of small mesenteric arteries from the same rats studied here. This previous study further indicated that a loss of vascular responsiveness following MI was not associated with increased ET-1 synthesis but is temporally linked to upregulation of ET_B receptors in the smooth muscle layer, which in turn is connected to inhibition of contraction via the ET_A receptor. Taken together with the results from the present study, these findings indicate that increased ET_B receptor mRNA expression is not just a phenomenon restricted to the heart, but is also observed in the vasculature.

Other investigators have reported an increase in cardiac ET_B receptor expression in conjunction with an increase in preproET-1 and ET_A receptor mRNA post-MI (Kobayashi *et al.*, 1999), although the cellular site of upregulation was not investigated. In a recent study, however, Smith *et al.* (in press) demonstrated selective upregulation of myocardial ET_B receptor mRNA in the non-infarcted LV of female ovariectomised rats 10-11 weeks post-MI. Interestingly, increased ET_B receptor mRNA was also localised to cardiomyocytes within the non-infarcted LV of these rats, which had an average infarct size of ~40% of the LV, similar to that found here. In the present study, increased ET_B receptor mRNA and immunoreactivity at 12 weeks post-MI was detected in the peri-infarct area of the non-infarcted LV, with little expression detected in remaining cardiomyocytes within the infarct and no expression detected at sites of deposited collagen at either time point, suggesting that ET_B receptors do not have a role in the scar at these time points. Furthermore, the lack of change in ET_B receptor expression in the RV, and the observation that lung weight was not significantly increased, suggests that increased ET_B receptor expression was not associated with RV hypertrophy at the time points studied, nor was increased ET_B expression a general phenomenon in the post-MI heart.

Two main questions therefore arise from this finding; 1) what causes the specific increase in ET_B receptors?, and 2) what role do the receptors have in the non-infarcted LV at this time? In response to the first question, a number of physiological

and/or hormonal factors may be involved in upregulation of ET_B receptors within the non-infarcted LV. A progressive increase in wall stress is associated with LV dysfunction during heart failure. Furthermore, a recent study by Morawietz *et al.* (2000) demonstrated upregulation of ET_B receptor mRNA by long-term arterial laminar stress in human umbilical vein endothelial cells *in vitro*. Although it is not known whether a similar effect is observed in cardiomyocytes *in vivo*, increased mechanical wall stress may be involved in regulation of ET_B receptor expression in these cells. In cultured cardiac myocytes, mechanical stretch can induce ET-1 expression (Yamazaki *et al.,* 1995), providing further evidence that increased mechanical stresses are a possible mechanism for the induction of the ET system in myocardium. However, that upregulated ET_B receptor mRNA expression alone was observed only at 12 weeks post-MI in the present study could be a characteristic of the MI model used, and may be a consequence of the progression from compensatory hypertrophy during LV dysfunction to decompensated heart failure. Furthermore, though this chapter only investigated the ET system up to a time of 12 weeks, upregulation of preproET-1 and ET_A mRNA may occur at a time after 12 weeks in this model.

Other mediators that may be involved in upregulation of ET_B receptors include the RAAS and norepinephrine, which are also increased during heart failure and can stimulate hypertrophy (Schunkert *et al.,* 1990; Kanno *et al.,* 1993; Colucci, 1997; Barth *et al.,* 2000). The relationship between ET-1 and the RAAS is noteworthy. Myocardial expression of angiotensin converting enzyme (ACE) is increased in rats with pressure overload ventricular hypertrophy and in the viable myocardium following experimental MI (Schunkert *et al.,* 1990; Callah *et al.,* 1995). Furthermore, Schunkert and colleagues demonstrated that the upregulation of ACE in the hypertrophied LV is associated with an increased intracardiac conversion of Ang I to Ang II (Schunkert *et al.,* 1993). Interestingly, in cultured cardiomyocytes, Ang II has been shown to induce the expression of mRNA for the ET_B receptor (Kanno *et al.,* 1993), thereby indicating a possible mechanism of increased ET_B receptor mRNA expression by upregulated Ang II in the non-infarcted LV. However, further tests are

needed to determine whether Ang II generation is increased in the same area as is ET_B receptor mRNA; specifically, the non-infarcted LV.

There may be several possible roles for upregulated ET_B receptors in the non-infarcted LV. In the present study, co-localisation of increased ET_B receptor expression with increased β-MHC immunoreactivity occurred in a specific area of the heart that may be subjected to increased progressive wall stress. As mentioned in Chapter 4, isoforms of cardiac MHC are altered in the failing myocardium of experimental CHF animals. Furthermore, the change from α-MHC to β-MHC is regarded as a molecular marker for hypertrophy in the failing myocardium (Michel *et al.,* 1988; Parker *et al.,* 1990b). ET-1 has also been shown to stimulate β-MHC production in ventricular myocardium *in vitro* (Wang *et al.,* 1992; Ichikawa *et al.,* 1996). Therefore, the fact that a significant upregulation of ET_B receptors was observed exclusively in an area of the heart where hypertrophy was specifically located suggests a potential role for ET_B receptors in regulation of processes such as hypertrophy and remodelling of the non-infarcted LV myocardium. Furthermore, ET-1 has been reported to exert hypertrophic effects in adult rabbit ventricular cardiomyocytes via stimulation of ET_B receptors (Mullan *et al.,* 1997).

During the past decade, significant progress has been made in identifying key intracellular signalling mechanisms that contribute to hypertrophic growth. These include protein kinase C, and the mitogen-activated protein kinase pathways, of which 3 major types exist: the extracellular signal-regulated protein kinase (ERK), c-jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase pathways (reviewed by Ruwhof & van der Laarse, 2000). In cardiac myocytes, the ERK pathway can be stimulated upon G-protein receptor occupation by binding of such peptides as ET-1 (Bogoyevitch *et al.,* 1994). Transcriptional activation of ERK was also shown to be modified by ET_B receptors (Aquilla *et al.,* 1996). Interestingly, upregulated ET_B receptor mRNA and increased ERK immunoreactivity were both found specifically in the non-infarcted LV at 12 weeks, but not 5 weeks, post-MI, suggesting further evidence of a role for ET_B receptors in regulating hypertrophy, possibly via an ERK-mediated intracellular pathway.

However, increased β-MHC staining was detected in the non-infarcted LV at 5 weeks post-MI in the present study, which does not match the time course for the observed upregulation in ET_B receptor expression, indicating that ET_B receptors may not have a role in initiation of the hypertrophic response. Nevertheless, it is possible that ET_B receptors may be involved in secondary processes that modulate hypertrophy that has been initiated earlier by other mediators such as TGF-βs or the RAAS, or, transiently, by ET-1 itself. Both TGF-βs (Li *et al.,* 1997) and the RAAS (reviewed by Wiollert *et al.,* 1999) have been shown to stimulate cardiac hypertrophy *in vitro*. Furthermore, TGF-β_1 immunoreactivity was found to be increased specifically in border myocytes in the non-infarcted LV at 5 and 12 weeks post-MI, with no changes in staining in the RV or septum. However, it is not known whether myocardial tissue levels of TGF-β_1 were sufficiently upregulated to induce hypertrophy in the rat model used in the present study.

A number of antagonist studies have investigated the role of ET receptors on hypertrophy post-MI. ET_A receptor antagonists have been shown to reduce LV hypertrophy and β-MHC expression, improve cardiac function, and increase survival following a large MI (Sakai *et al.*, 1996a, 1996b, 1998), despite the fact that ET_B receptors are also upregulated in the same model (Kobayashi *et al.,* 1999). However, in a less severe model, with smaller infarcts, treatment with the ET_A receptor antagonist had no effect on LV hypertrophy (Mulder *et al.,* 1998), while the non-selective ET receptor antagonist bosentan reduced LV hypertrophy (Mulder *et al.,* 1997) or LV dilatation (Fraccarollo *et al.,* 1997). The results of the present study showing an increase in ET_B receptor mRNA expression with no change in ET_A receptor or preproET-1 mRNA expression after moderate MI may therefore help explain the above observations, outlining the importance of mixed ET receptor antagonism after moderate MI. This may not only be the case when LV dysfunction is caused by MI. A recent study reported that ET_A receptor antagonism improved LV dysfunction in severe but not mild heart failure induced by rapid ventricular pacing (Schirger *et al.,* 1999).

Only one antagonist study has specifically investigated the role of ET_B receptors post-MI *in vivo*. Mulder *et al.* (2000) recently showed that chronic, simultaneous blockade of both ET_A and ET_B receptors improved systemic and cardiac haemodynamics, as well as LV function and remodelling, to the same extent as ET_A receptor blockade alone in a rat CHF model after moderate MI. However, these effects were associated with a significant reduction of heart rate only with simultaneous ET_A-ET_B receptor blockade. Furthermore, chronic, selective ET_B receptor blockade per se, while having no adverse haemodynamic effects or showing functional improvement, reduced cardiac collagen accumulation.

The fact that no increased plasma ET-1 levels or adverse haemodynamic effects were observed with chronic selective ET_B receptor or simultaneous ET_A and ET_B receptor antagonism in the above study is very interesting. The effects of chronic ET_B receptor antagonism were previously unknown, though in theory, the blockade of ET_B receptors may have deleterious effects, either by reducing ET_B-mediated endothelium-dependent vasodilation or by decreasing ET-1 clearance (Fukuroda *et al.,* 1994) and thus increasing plasma ET-1 levels. In Chapter 3, administration of a non-selective ET receptor antagonist to post-MI rats for 2 weeks led to a specific increase in plasma ET-1 levels. However, that chronic treatment (over 4 weeks) led to no increased elevated plasma ET-1 levels or adverse haemodynamic effects (Mulder *et al.,* 2000) suggests that plasma ET-1 may be cleared by an alternative mechanism during long-term ET_B receptor blockade and that chronic ET_B receptor antagonism may be tolerated as a potential therapy against heart failure.

Upregulated ET_B receptors may alternatively have other roles in the non-infarcted LV such as mediating contractility or rhythmicity of the myocardium (Molenaar *et al.,* 1993; Miyauchi & Masaki, 1999). Mulder *et al.* (2000) showed that long-term combined ET_A/ET_B receptor blockade in a rat chronic heart failure model led to a significant decrease in heart rate compared to long-term via specific ET_A receptor inhibition, indicating a potential role for the ET_B receptor in this process.

Conversely, ET_B can transmit growth inhibitory effects associated with apoptosis (Okazawa *et al.,* 1998). Cardiomyocyte-specific apoptosis was shown to contribute

to the transition from LV hypertrophy to LV dysfunction (Condorelli *et al.,* 1999). However ERK has been shown to protect cardiomyocytes from apoptosis (Aikawa *et al.,* 1997). The balance between these influences and interactions with other systems such as the RAAS and progressive physiological wall stress may therefore determine the course of myocardial remodelling during heart failure.

In conclusion, the association of increased ET_B receptor mRNA with growth regulatory genes suggests a role for ET_B receptors in modulation of the LV response to MI, although further studies using ET receptor antagonists are required to confirm their specific role. Furthermore, the above antagonist studies along with the present study together indicate the importance of infarct size in activation of the ET system post-MI.

Chapter 6

General Discussion

Heart failure is a major and increasing cause of cardiovascular morbidity and mortality, due at least in part to the fact that its various haemodynamic, neuroendocrine and other pathological mechanisms remain incompletely understood. The introduction of the angiotensin-converting enzyme (ACE) inhibitors has clearly improved symptoms and decreased mortality, but established heart failure still impairs quality of life more than any other chronic illness (Dargie & McMurray, 1994). ET-1 has been implicated in the pathophysiology of CHF with increasing plasma concentrations correlating with the degree of haemodynamic and functional impairment. Furthermore, there are many similarities between ET-1 and the RAAS such as their vasoconstrictor, mitogenic and anti-natriuretic properties, all of which aggravate progression of CHF. At the onset of this thesis, the distribution of the local myocardial ET system within the normal heart, and if, when, or where components of the ET system may change during the development of heart failure following MI had not been investigated.

In this thesis, I set out to initially characterise the ET-1 system in healthy hearts of rats with reference to ET-1 peptide distribution and cellular localisation in the myocardium and coronary vasculature. Another aim was to investigate any involvement of the myocardial ET-1 system during scar formation following MI induced by coronary artery ligation (CAL) in the rat. Finally the myocardial ET-1 system was studied with reference to myocardial hypertrophy and fibrosis later post-MI. In summary, I set out to further investigate the myocardial ET-1 system as a viable target for future drug therapy for heart failure. By investigating changes in regulation of the ET-1 system both early and later post-MI, I hoped to identify the possible type of antagonism and the times at which potential therapy may be most beneficial.

The primary finding of this thesis was the evidence of ET-1 peptide present in the normal rat heart. ET-1 immunoreactivity could be located to cardiomyocytes, myoendothelial cells and some coronary vascular endothelial cells within myocardium from normal and sham-op rats indicating the presence of mature ET-1 peptide in these cells under normal physiological conditions. This finding agrees

with recent research showing ET-1 immunoreactivity throughout the myocardium of control group rat hearts from MI studies (Sakai *et al.,* 1996b; Øie *et al.,* 1997; Tønnessen *et al.,* 1998).

That ET-1 immunoreactivity was observed within the myocardium does not confirm on its own the presence of a local myocardial ET-1 system. ET-1 immunoreactivity may be highlighting bound ET-1 peptide, which may have been produced away from the heart and taken up by myocardial tissue from circulating plasma. However, *in situ* hybridisation studies using sham-op rat hearts further showed that preproET-1 mRNA was widespread throughout all 4 chambers of the heart and associated with cardiomyocytes and the endothelium of coronary blood vessels. ET_A receptor mRNA and, to a much lesser extent, ET_B receptor mRNA was also detected throughout the myocardium. These results complement the finding of ET-1 peptide immunoreactivity throughout the myocardium and confirm previous suggestions of a local cardiac ET system in the heart with the ability to synthesise, and be activated by, ET-1 (Suzuki *et al.,* 1993; Molenaar *et al.,* 1993; Evans *et al.,* 1994; Plumpton *et al.,* 1996). Furthermore, it extends these studies by localising the distribution of preproET-1, ET_A receptor and ET_B receptor mRNA to cardiomyocytes throughout the myocardium.

Endogenous generation of ET-1 was shown to contribute to basal vascular tone in healthy humans (Haynes & Webb, 1994), and also to regulation of basal coronary tone in the rat isolated heart (Goodwin *et al.,* 1998). Recent studies have also indicated a role for the local cardiac ET system in contributing to contractility in the normal healthy heart. Kelso *et al.* (2000) reported that the ET_A receptor subtype, and not the ET_B receptor subtype, mediates the positive inotropic effect of ET-1 in LV cardiomyocytes isolated from the rabbit heart. Furthermore, MacCarthy *et al.* (2000) reported that endogenous ET-1 has a tonic positive inotropic effect on cardiac contraction in normal subjects, independent of the effects of the peripheral vasculature, and is inhibited by blockade of the ET_A receptors. In summary, these studies along with the present data indicate the presence of a local myocardial ET system, which has a role in regulation of physiological effects within the heart.

The next finding in this thesis indicated transient regulation of myocardial ET-1 within the infarct during scar formation post-MI. After CAL surgery in the rat, ET-1 immunoreactivity was shown to increase within the developing scar over the first 14 days post-MI, with the number of ET-1 staining cells within the infarct peaking at 7 days. ET-1 immunoreactivity could be localised in remaining cardiomyocytes and areas of granulation, with staining present in the cytoplasm of inflammatory cells involved in clearance of necrotic tissue, fibroblasts synthesising collagen and endothelial cells involved in new vessel formation. Furthermore, fibroblasts, endothelial cells, and inflammatory cells within granulation tissue showing ET-1 immunoreactivity at 7 days post-MI, also showed TGF-β_1 immunoreactivity. Other recent studies have also shown increased ET-1 staining in the scar early post-MI with staining localised to similar cells as mentioned above (Øie *et al.,* 1997; Tønnessen *et al.,* 1998). Since there were no changes in plasma ET-1 concentrations at either the 2, 7 or 14-day times studied, these results suggested that locally synthesised ET-1 was upregulated within the infarct early post-MI and was present in cells involved in active processes of scar formation. However, preproET-1 mRNA expression was not measured in this part of the study and the possibility that there is increased uptake of plasma ET-1 from the circulation into the infarcted region cannot be excluded.

This question of whether upregulated ET-1 within the infarct was beneficial or detrimental in scar formation was addressed in Chapter 4, which investigated the role of upregulated ET-1 within the developing scar using a non-selective ET receptor antagonist A-182086 (30mg/kg/day) administered to rats upon recovery from CAL surgery, and continued daily for the duration of the study. Results from this study indicated a reduced TUNEL-positive cell count within the infarct of the 2-day CAL+A-182086 group, a decreased heart weight within the 14-day CAL+A-18086 group and a significant increase in myocardial content within the infarct of hearts from rats in the 7 and 14-day CAL+A-18086 groups. However, where areas of granulation were observed in the infarct area, active processes of scar formation including inflammation, collagen synthesis, TGF-β_1 activation and new vessel formation were still evident between 2 and 14 days post-CAL, indicating that blockade of ET receptors was not sufficient to halt these processes. Furthermore, 2

CAL + A-182086 group rats (1 from the 7-day group and 1 from the 14-day group) that did not eat their food over the first 24 hours, but ate daily thereafter for the duration of the study, developed thinned defined infarcts with little myocardial content. This implies that antagonist–mediated improved myocardial cell content within the infarct was a phenomenon related to the first 24 hours of treatment.

As mentioned in the discussion of Chapter 4, synthesis and release of myocardial ET-1 is increased during hypoxia *in vitro* and plasma levels are transiently elevated post-MI. The action of non-selective ET receptor antagonism administered immediately upon recovery from CAL may, therefore, be inhibiting an acute, transient, ET-1-mediated vasoconstriction, which further reduces any remaining blood flow or collateral circulation into or around the affected area. The fact that heart weight was lower at 14 days in the CAL + A-18086 group, along with reduced β-MHC immunoreactivity in the non-infarcted left ventricle (LV) and salvaged myocardium of the infarct, may be due to the increased myocardial content within the infarct lowering wall stress in the LV. This would therefore reduce the workload in remaining cardiomyocytes and any compensatory hypertrophic response in the LV. Overall, these findings indicate that early administration of a non-selective ET receptor antagonist increases myocardial content within the infarct, which may in turn reduce ventricular remodelling by lowering workload in the remaining LV myocardium. Furthermore, upregulated ET-1 within the developing scar at 7 and 14 days post-MI does not appear to be critical in regulating the active processes of scar formation.

Having considered the results, there are now several additional studies I would like to perform to strengthen the thesis and enhance the understanding of the role of the myocardial ET-1 system post-MI. Further experiments are required to investigate whether an acute, transient, increase in myocardial ET-1 levels is observed over the first 48 hours after surgery. Plasma ET-1 levels could be measured along with investigation of ET-1 immunoreactivity within the infarct (ET-1 staining within the infarct was sparse at 2 days post-MI) and *in situ* hybridisation studies highlighting preproET-1 mRNA levels. These data would help to clarify whether myocardial

necrosis within the infarct is further augmented by acute, transient, increases in myocardial ET-1 levels, and that the benefits of early non-selective antagonism are mediated by blocking this effect.

The salvaged myocardium within infarcts of hearts from the CAL + A-182086 group rats showed a cellular morphology, vascular supply and ET-1 immunoreactivity similar to normal myocardium. However, actual tissue protein levels of ET-1 peptide or expression levels of preproET-1, ET_A and ET_B receptor mRNA from specific areas of the heart could not be measured. Acquiring these data via reverse transcription polymerase chain reaction (rtPCR) and *in situ* hybridisation studies would confirm my results indicating viable salvaged myocardium with the ability to express and synthesis ET-1. It is also not known whether the salvaged myocardium retains its functional capacity. A recent review stated that the scar formed after infarction has the ability to contract through the presence of myofibroblasts (Sun & Weber, 2000). Hence, future functional studies could be performed, for example using echocardiography, to identify whether contractility of the LV wall improves after ET antagonist treatment. Furthermore, whether early antagonist treatment improves myocardial remodelling within the LV in the long-term requires further investigation via additional chronic antagonist studies.

Endothelin converting enzyme (ECE)-1 is an important component of the ET-1 synthesis pathway (see section 1.2.2). Studies investigating ECE post-MI have found no change in ECE-1 mRNA or ECE-1 immunoreactivity (Kobayashi *et al.,* 1999) in the rat model of MI. However, increased ECE-1 mRNA has been measured in arterial tissue in human cardiovascular disease (Bohnemeier *et al.,* 1998). Furthermore, it is not known whether expression of ECE-1 changes, or has an important regulatory role, within the infarct during scar formation early post-MI. It would, therefore, be interesting to characterise ECE (immunohistochemically and via *in situ* hybridisation) to determine whether upregulation occurs along with increased ET-1 during scar formation. Also, with the advent of new potent non-peptide inhibitors of ECE-1 (De Lombaert *et al.,* 2000), it would be interesting to compare

the effects of an ECE inhibitor to those found using a non-selective ET receptor antagonist in the present study.

The involvement of locally produced angiotensin (Ang) II in tissue repair post-MI (reviewed by Sun & Weber, 2000) and in the pathophysiology of cardiac hypertrophy (Wollert *et al.,* 1999) suggests an important role for this peptide in heart failure. Ang II has been shown to stimulate cardiomyocyte hypertrophy via paracrine release of ET-1 and TGF-β_1 from fibroblasts *in vitro* (Gray *et al.,* 1998). The fact that many active processes of scar formation including collagen synthesis, inflammation, angiogenesis and TGF-β_1 activation are observed in granulation tissue suggests that Ang II may have an important role in these processes outwith the presence or influence of ET-1. It would be interesting, therefore, to investigate the ET-1 system in hearts from rats treated with an ACE inhibitor or an AT_1 receptor antagonist post-CAL, to identify whether increased ET-1 immunoreactivity is a consequence of increased stimulation of ET-1 synthesis by Ang II. It would also be interesting to investigate Ang II immunoreactivity in hearts from the A-182086-treated and untreated CAL groups to identify whether non-selective ET receptor antagonism affects regulation of the local myocardial RAAS early post-MI.

Chapter 5 investigated the myocardial ET-1 system at later times post-MI in the rat with specific reference to preproET-1, ET_A and ET_B receptor mRNA expression. Unfortunately, due to problems in measuring some plasma ET-1 and big ET-1 values and heart weights, and without LVEDP measurements, interpretation of these data in confirming CHF per se was not possible in the 5- and 12-week post-MI groups, and is an obvious weakness in this study. Nevertheless, CAL surgery did produce rats with moderate sized infarcts (~40-50% of the LV free wall), indicating, at least, a rat model with LV dysfunction.

At 5 and 12 weeks post-MI, expression of preproET-1, ET_A receptor and ET_B receptor mRNA was significantly reduced within the infarct. Furthermore, there were no changes in either preproET-1 or ET_A receptor mRNA at both times, in the non-infarcted LV, RV or septum. These results are in agreement with other studies using

rats with similar sized infarcts at 6 weeks post-MI (Øie *et al.,* 1997, Tønnessen *et al.,* 1997) and contrast with studies using rats with much larger infarcts where upregulated expression of the ET-1 system was observed in the LV at 3 (Sakai *et al.*, 1996a; Kobayashi *et al.,* 1999) and 12 (Sakai *et al.*, 1996b) weeks post-MI. Overall, this suggests a relationship between the severity of infarction and activation of the myocardial ET system post-MI.

At 12 weeks, but not 5 weeks, a profound increase in ET_B receptor expression was observed in the non-infarcted LV only, associating with an increase in ERK immunoreactivity and a progressive increase in β-MHC staining. The fact that the significant upregulation of ET_B receptors was observed exclusively in an area of the heart where hypertrophy and increased ERK were located suggest a specific role for ET_B receptors in regulation of processes such as hypertrophy and remodelling of the non-infarcted LV myocardium. Therefore, in this moderate MI model, ET_B receptors may be involved in maintaining a hypertrophic response in the heart during changes from compensatory to decompensated heart failure. Further studies are required, however, to clarify whether a direct association exists between increased ET_B receptor expression and increased ERK activation and β-MHC immunoreactivity within the non-infarcted LV. A number of situations are possible whereby a stimulating factor may increase ET_B receptor mRNA expression and activate ERK independently of each other. Also, whether β-MHC is further increased as a consequence of increased ET_B receptor expression or whether increased β-MHC immunoreactivity is independent of increased ET_B receptor expression is unclear.

The above experiments may be investigated using ET receptor antagonists. Since changes in ET_B receptor expression were observed at 12 weeks and not 5 weeks post-MI, it would be interesting, therefore, to investigate changes in hypertrophy and remodelling within the LV of rat hearts administered either a non-selective ET, or a selective ET_A, receptor antagonist at 5 weeks post-MI. A recent paper reported that late treatment with either an AT_1 receptor blocker or an ACE inhibitor in spontaneously hypertensive rats, between 18 and 22 months of age, reversed myocyte hypertrophy indicating that it is possible to significantly reverse

remodelling pharmacologically even if therapy is initiated near the onset of failure (Tamura *et al.*, 2000). Comparing the findings from separate non-selective ET, and ET_A selective, antagonist studies would clarify whether specific ET_B receptor upregulation was beneficial or detrimental to changes within the LV during progression of heart failure. It would also identify whether ET-1 is involved in increased activation of ERK via an upregulated ET_B receptor-mediated pathway.

Clinical implications

In the present study, a potential therapeutic role for non-selective ET receptor antagonism has been identified in the acute phase after MI. Administration of A-182086 led to an increased myocardial content within the infarct, lower heart weights and reduced β-MHC staining, which may have beneficial effects on hypertrophy and ventricular remodelling that contribute to the development of CHF. These effects may be mediated through an indirect effect of ET receptor antagonism on an acute ET-1 mediated vasoconstriction within the LV induced via hypoxia and/or irreversible cell damage. Furthermore, a similar study using a selective ET_A receptor antagonist reported aggravated chronic LV remodelling with no improvement in haemodynamics (Hu *et al.*, 1998), indicating the therapeutic potential of a non-selective antagonist over selective ET_A receptor antagonism during post-MI scar formation.

Interestingly, selective ET_B receptor mRNA expression is upregulated specifically in the non-infarcted LV at 12 weeks post-MI in the rat model subjected to moderate MI. Whether upregulated ET_B receptors are directly involved in regulation of hypertrophy at a later time point in heart failure is of great interest, and may indicate a beneficial choice of using a non-selective ET receptor antagonist over that of a selective ET_A receptor antagonist as a potential therapy during this time. Recent clinical research in heart failure has identified a therapeutic role for ET receptor antagonism during the later stages of CHF. The REACH-1 trial (reviewed by Mylona & Cleland, 1999) reported improvements in symptoms using the non-selective ET receptor antagonist bosentan (500mg BID), though an increase in hepatic

transaminases led to a premature halt in the study. However, a follow-up study found that a lower concentration of bosentan (125mg BID, Krum *et al.*, 1999) was well tolerated and maintained the clinical improvement observed in REACH-1. The use of selective ET_B receptor antagonism in the clinical setting remains uncertain at present. Only one study to date has investigated chronic selective ET_B receptor antagonism in the rat CHF model, which indicated no adverse effects and reduced cardiac collagen accumulation (Mulder *et al.*, 2000). Suprisingly, an increase in plasma ET-1 levels was not observed. However, in the same study, simultaneous administration of a selective ET_A and ET_B receptor antagonist improved systemic and cardiac haemodynamics, as well as LV function and remodelling, to the same extent as ET_A receptor blockade alone. In addition, a significant reduction of heart rate was associated only with simultaneous ET_A-ET_B receptor blockade. These findings suggest potential therapeutic benefits of using a non-selective ET receptor antagonist compared to selective ET_A or ET_B receptor antagonists alone in a rat model of CHF after moderate MI.

Another major finding of this thesis was the relationship between infarct size and activation of the ET-1 system post-MI. Therefore, further studies need to be performed to identify whether selective ET receptor antagonism is required for the varied pathological states of CHF.

Conclusion

In conclusion, this thesis has indicated transient upregulation of the ET-1 system during scar development early post-MI and specific regulation of ET receptors related to severity later post-MI. Although further research is required in both the laboratory and at the clinical level to complement findings in the present study, the present study indicates that acute and/or chronic targeting of the ET-1 system may prove beneficial in the fight towards improving symptoms, reducing hospital admissions and lowering the economic burden that heart failure places on society today.

Appendix

All salts were purchased from BDH, UK.

Buffers

Phosphate Buffered Solution (PBS) pH 7.6

1. Dissolve 12.7g di-sodium hydrogen orthophosphate in 80 ml deionised H_2O. Microwave for 30 seconds.
2. In a separate container, dissolve 1.7g of sodium di-hydrogen orthophosphate in 80 ml deionised H_2O.
3. Add both solutions to 800 ml deionised H_2O and pH to 7.6 with concentrated HCl.
4. Make up final volume to 1L with deionised H_2O.

Tris-Buffered Solution (TBS) pH 7.8

1. Dissolve 6.04g of tris in 80 ml deionised H_2O. Add 2.77 ml of concentrated HCl.
2. In a separate container, dissolve 8.1 g of sodium chloride in 900 ml deionised H_2O.
3. Mix the 2 solutions, adjust to pH 7.8 using concentrated HCl and make up to 1L.

10xPBS for in situ

1. Dissolve 80g of sodium chloride, 2g of potassium chloride, 29g of sodium di-hydrogen orthophosphate, and 2g of potassium di-hydrogen orthophosphate in 900ml deionised H_2O.
2. Adjust volume to 1L and autoclave.

Tris-EDTA (TE) Buffer for in situ.

1. Mix 5 ml of 10mM tris chloride (pH 8.0) with 2 ml of 1mM EDTA (pH 8.0).

Prehybridisation Buffer

The following substances were added to 2.94 ml of deionised H_2O:

1. 1.2 ml of 5M sodium chloride
2. 100µl of 1M tris (pH 7.5)
3. 40µl of 250mM EDTA
4. 500µl of 10mg/ml salmon sperm DNA (Sigma, UK)
5. 20µl of 50mg/ml yeast tRNA (Sigma, UK), make up to 500ml with deionised H_2O
6. 200µl of denhardt's (Sigma, UK)

Radioimmunoassay buffer

To 1L of deionised H_2O the following compounds were added:

1. 3.853g of sodium dihydrogen phosphate
2. 18.07g of disodiumhydrogen phosphate
3. 2.927g of sodium chloride
4. 1g bovine serum albumen (Sigma, UK)
5. 100mg sodium azide (BDH, UK)
6. 1 ml Triton X-100 (BDH, UK)

4% Paraformaldehyde

1. Dissolve 2.4g of sodium di-hydrogen orthophoshate and 11.36g of di-sodium hydrogen orthophosphate in 1L of DEP H_2O.
2. Heat to approximately 80°C, then add 40g paraformaldehyde (BDH, UK).
3. Stir for 1 hour at approximately 80°C, then store at 4°C.
   Paraformaldehyde can be stored for 2 weeks.

Stains

Van Gieson's stain

To 50ml of distilled H_2O, add:

1. 50ml of saturated aqueous picric acid solution (BDH, UK)
2. 9ml of 1% aqueous acid fuschin solution (BDH, UK)

Aminopropylethoxysaline (TESPA) Slide Coating

Bathe slides for 10 seconds in each of the following:

1. 10% HCl in 70% ethanol
2. DEP H_2O
3. 100% acetone

Air dry the slides, then 10 seconds in each of the following:

4. 2% TESPA in acetone
5. 100% acetone
6. 100% acetone

Slides are air dried again, can be stored for one month in airtight containers.

References

Agocha A., Lee H.W. & Eghbali-Webb M. (1997) Hypoxia regulates basal and induced DNA synthesis and collagen type I production in human cardiac fibroblasts: Effects of transforming growth factor beta (1), thyroid hormone, angiotensin II and basic fibroblast growth factor. *J. Mol. Cell. Cardiol.* **29,** 2233-2244.

Aikawa R., Komuro I., Yamazaki T. *et al.* (1997) Oxidative stress activates extracellular signal-regulated kinases through Src and Ras in cultured cardiac myocytes of neonatal rats. *J. Clin. Invest.* **100,** 1813-1821.

Allcock G.H., Warner T.D. & Vane J.R. (1993) The endothelin receptor antagonists BQ-123 and PD-145065 reverse established vasoconstrictor responses to endothelin-1 *in vitro*. *Br. J. Pharmacol.* **110,** 150.

Andersson B. & Waagstein F. (1993) The spectrum and outcome of congestive-heart-failure in a hospitalised population. *Circulation* **88,** 358-358.

Anversa P., Cheng W., Liu Y., Leri A., Redaelli G. & Kajstura J. (1998) Apoptosis and myocardial infarction. *Basic Res. Cardiol.* **93,** 8-12.

Apps D.K., Cohen B.B. & Steel C.M. (1992) Clinical Enzymology. In: *Biochemistry: A Concise Text for Medical Students.* (eds Apps D.K., Cohen B.B. & Steel C.M.), 5th edn, pp. 52-54. Bailliere Tindal.

Aquilla E., Whelchel A., Knot H.J., Nelson M. & Posada J. (1996) Activation of multiple mitogen-activated protein kinase signal transduction pathways by the endothelin B receptor requires the cytoplasmic tail. *J. Biol. Chem.* **271,** 31572-31579.

Arai H., Hori S., Aramori I., Ohkubo H. & Nakanishi S. (1990) Cloning and expression of a cDNA-encoding an endothelin receptor. *Nature* **348,** 730-732.

Asano K., Dutcher D.L., Port J.D. *et al.* (1997) Selective downregulation of the angiotensin II AT (1)-receptor subtype in failing human ventricular myocardium. *Circulation* **95,** 1193-1200.

Bakris G., Bursztyn M., Gavras I., Bresnahan M. & Gavras H. (1997) Role of vasopressin in essential hypertension: Racial differences. *J. Hypertens.* **15,** 545-550.

Barnes K., Shimada K., Takahashi M., Tanzawa K. & Turner A.J. (1996) Metallopeptidase inhibitors induce an upregulation of endothelin converting enzyme levels and its redistribution from the plasma membrane to an intracellular compartment. *J. Cell Sci.* **109,** 919-928.

Barth W., Deten A., Bauer M. *et al.* (2000) Differential remodelling of the left and right heart after norepinephrine treatment in rats: studies on cytokines and collagen. *J. Mol. Cell. Cardiol.* **32,** 273-284.

Batra V.K., Mcneilj J.R., Xu Y.J., Wilson T.W. & Gopala-Krishnan V. (1993) ET_B receptors on aortic smooth muscle cells of spontaneously hypertensive rats. *Am. J. Physiol.* **264,** C479-C484.

Battistini B., Chailler P., Dorleansjuste P., Briere N. & Sirois P. (1993) Growth regulatory properties of endothelins. *Peptides* **14,** 385-399.

Bauersachs D., Fraccarollo D., Hu K. *et al.* (1998) Chronic ischaemic heart failure in the rat increases vascular superoxide production by an NADH-dependent oxidase: contribution to endothelial dysfunction. *Circulation* **98,** 3851.

Bax W.A., Bruinvels A.T., van Sylen R.J., Saxena P.R. & Hoyer D. (1993) Endothelin receptors in the human coronary artery, ventricle and atrium: a quantitative autoradiographic analysis. *Arch. Pharmacol.* **348,** 403-410.

Bek E.L. & McMillen M.A. (1999) Endothelin receptor A mediates endothelin-induced angiogenesis. *FASEB J.* **13,** A362.

Belloni A.S., Andreis P.G., Neri G. & Nussdorfer G.G. (1995) Endothelin-1 (ET-1) and cyclosporine-A (CsA) stimulate steroid secretion from rat adrenal cortex - evidence that both ET-1 and CsA secretagogue effects are mediated by the B-subtype of ET-1 receptors. *Biomed. Res.-Tokyo* **16,** 287-294.

Benigni A. (2000) Endothelin antagonists in renal disease. *Kid. Int.* **57,** 1778-1794.

Berridge M.J. (1993) Inositol trisphosphate and calcium signalling. *Nature* **361,** 315-325.

Bishop J.E., Greenbaum R., Gibson D.G., Yacoub M. & Laurent G.J. (1990) Enhanced deposition of predominantly type-I collagen in myocardial disease. *J. Mol. Cell. Cardiol.* **22,** 1157-1165.

Bishorpic N.H., Simpson P.C. & Ordahl C.P. (1987) Induction of the skeletal alpha-actin gene in alpha-1 adrenoreceptor-mediated hypertrophy of rat cardiomyocytes. *J. Clin. Invest.* **80,** 1194.

Bogoyevitch M.A., Glennon P.E., Andersson M.B. *et al.* (1994) Endothelin-1 and fibroblast growth factors stimulate the mitogen-activated protein kinase signalling cascade in cardiac myocytes - the potential role of the cascade in the integration of 2 signalling pathways leading to myocyte hypertrophy. *J. Biol. Chem.* **269,** 1110-1119.

Bohnemeier H., Pinto Y., Horkay F. *et al.* (2000) Endothelin converting enzyme-1 mRNA expression in human cardiovascular disease. *Clin. Exp. Hypertens.* **20,** 417-437.

Bokil H.S., Porter J.P. & Joshua I.G. (1999) Decreased endothelin receptor (ET_B) mRNA expression in aortic tissue of DOCA-salt hypertension. *FASEB J.* **13,** A112.

Bonin P.D., Leadley R.J. & Erickson L.A. (1993) Growth factor induced modulation of endothelin-1 binding to human smooth muscle cells. *J. Cardiovasc. Pharmacol.* **22,** S125-S127.

Bonvallet S.T., Oka M., Yano M. *et al.* (1993) BQ-123, an ET (A) Receptor antagonist, attenuates endothelin-1-induced vasoconstriction in rat pulmonary circulation. *J. Cardiovasc. Pharmacol.* **22,** 39-43.

Bremnes T., Paasche J.D., Mehlum A. *et al.* (2000) Regulation and intracellular trafficking pathways of endothelin receptors. *J. Biol. Chem.* **275,** 17596-17604.

Brown L.A., Nunez D.J., Brookes C.I.O. & Wilkins M.R. (1995) Selective increase in endothelin-1 and endothelin-A receptor subtype in the hypertrophied myocardium of the aorto-venacaval fistula rat. *Cardiovasc. Res.* **29,** 768-774.

Brunner F., Dutoit E.F., & Opie L.H. (1992) Endothelin release during ischemia and reperfusion of isolated perfused rat hearts. *J. Mol. Cell. Cardiol.* **24,** 1291-1305.

Brunner F. & Opie L.H. (1996) Endothelin subtype A receptors mediate endothelin-1 effects on ischaemic contracture and reperfusion function in the rat. *Circulation* **94,** 3550.

Bugajski P., Kalawski R., Balinski M. *et al.* (1999) Plasma-mediated stimulation of neutrophil superoxide anion production during coronary artery bypass grafting: Role of endothelin-1. *Thorac. Cardiovasc. Surg.* **47,** 144-147

Bugge E. & Ytrehus K. (1996) Endothelin-1 can reduce infarct size through protein kinase C and K- ATP channels in the isolated rat heart. *Cardiovasc. Res.* **32,** 920-929.

Buja L.M. & Entman M.L. (1998) Modes of myocardial cell injury and cell death in ischaemic heart disease. *Circulation* **98,** 1355-1357.

Burke S.E. & Nelson R.A. (1997) Endothelin-receptor antagonist FR 139317 reduces infarct size in a rabbit model when given before, but not after, coronary artery occlusion. *J. Cardiovasc. Pharmacol.* **29,** 87-92.

Burkhardt M., Barton M. & Shaw S.G. (2000) Receptor- and non-receptor-mediated clearance of big-endothelin and endothelin-1: different effects of acute and chronic ET_A receptor blockade. *J. Hypert.* **18,** 273-279.

Burrell K.M., Molenaar P., Dawson P.J. & Kaumann A.J. (2000) Contractile and arrhythmic effects of endothelin receptor agonists in human heart *in vitro*: Blockade with SB 209670. *J. Pharmacol. Exp. Therapeut.* **292,** 449-459.

Burrell L.M., Risvanis J., Johnston C.I., Naitoh M. & Balding L.C. (2000) Vasopressin receptor antagonism -a therapeutic option in heart failure and hypertension. *Exp. Physiol.* **85,** S259-S265.

Callah M., Nicoletti A., Arnal J.F. *et al.* (1995) Cardiac angiotensin converting enzyme overproduction indicates interstitial activation in renovascular hypertension. *Cardiovasc. Res.* **30,** 231-239.

Cannan C.R., Burnett J.J.C. & Lerman A. (1996) Enhanced coronary vasoconstriction to endothelin-B receptor activation in experimental congestive heart failure. *Circulation* **93,** 646-651.

Cao L. & Gardner D.G. (1995) Natriuretic peptides inhibit DNA-synthesis in cardiac fibroblasts. *Hypertension* **25,** 227-234.

Carey R.M., Jin X.H., Wang Z.Q. & Siragy H.M. (2000) Nitric oxide: a physiological mediator of the type 2 (AT(2)) angiotensin receptor. *Acta Physiol. Scand.* **168,** 65-71.

Cargnelli G., Rossi G.P., Pessina A.C. *et al.* (1998) Changes of blood pressure and aortic strip contractile responses to ET-1 of heterozygous female transgenic rats, TGR(mRen2)27. *Pharmacol. Res.* **37,** 207-211.

Casscells W., Bazoberry F., Speir E. *et al.* (1990) Transforming growth factor-beta-1 in normal heart and in myocardial-infarction. *Ann. New York Acad. Sci.* **593,** 148-160.

Cavero P.G., Miller W.L., Heublein D.M., Aarhus L.L. & Burnett J.C.J. (1990) Endothelin in experimental congestive heart failure in the anaesthetised dog. *Am.J.Physiol. (Renal Fluid Electrolyte Physiol.)* **259,** F312-F317.

Chanez P., Vignola A.M., Albat B. *et al.* (1996) Involvement of endothelin in mononuclear phagocyte inflammation in asthma. *J. All. Clin. Immunol.* **98,** 412-420.

Charney R. & Cohen M. (1993) The role of the coronary collateral circulation in limiting myocardial ischaemia and infarct size. *Am. Heart. J.* **126,** 937-945.

Cheng W., Kajstura J., Nitahara J.A. *et al.* (1996) Programmed cell death affects the viable myocardium after myocardial infarction in rats. *Exp. Cell Res.* **226,** 316-327.

Clerk A. & Sugden P.H. (1997) Regulation of phospholipases C and D in rat ventricular myocytes: Stimulation by endothelin-1, bradykinin and phenylephrine. *J. Mol. Cell. Cardiol.* **29,** 1593-1604.

Cleutjens J.P.M., Verluyten M.J.A., Smits J.F.M. & Daemen M.J.A.P. (1995) Collagen remodelling after myocardial-infarction in the rat-heart. *Am. J. Pathol.* **147,** 325-338.

Cleutjens J.P.M., Blankesteijn W.M., Daemen M.J.A.P. *et al.* (1999) The infarcted myocardium: Simply dead tissue, or a lively target for therapeutic interventions. *Cardiovasc. Res.* **44,** 232-241.

Clozel M., Gray G.A., Breu V., Loffler B.M. & Osterwalder R. (1992) The endothelin et(B) Receptor mediates both vasodilation and vasoconstriction *in vivo*. *Biochem. Biophys. Res. Commun.* **186,** 867-873.

Coghlan J.P., Aldred P., Haralambidis J. *et al.* (1985) Hybridisation histochemsitry. *Anal. Biochem.* **149,** 1-28.

Colucci W.S. (1997) Molecular and cellular mechanisms of myocardial failure. *Am. J. Cardiol.* **80** [11A], 15L-25L.

Condorelli G., Morisco C., Stassi G. *et al.* (1999) Increased cardiomyocyte apoptosis and changes in pro-apoptotic and anti-apoptotic genes bax and bcl-2 during left ventricular adaptations to chronic pressure overload in the rat. *Circulation* **99,** 3071-3078.

Corder R. & Barker S. (1999) The expression of endothelin-1 and endothelin-converting enzyme-1 (ECE-1) are independently regulated in bovine aortic endothelial cells. *J. Cardiovasc. Pharmacol.* **33,** 671-677.

Crawford D.C., Chobanian A.V. & Brecher P. (1994) Angiotensin-II induces fibronectin expression associated with cardiac fibrosis in the rat. *Circ. Res.* **74,** 727-739.

Cristol J.P., Warner T.D., Thiemermann C. & Vane J.R. (1993) Mediation via different receptors of the vasoconstrictor effects of endothelins and sarafotoxins in the systemic circulation and renal vasculature of the anaesthetised rat. *Br. J. Pharmacol.* **108,** 776-779.

Croft J.B., Giles W.H., Pollard R.A. *et al.* (1999) Heart failure survival among older adults in the United States - A poor prognosis for an emerging epidemic in the Medicare population. *Arch. Intern. Med.* **159,** 505-510.

Cromack D.T., Sporn M.B., Roberts A.B. *et al.* (1987) Transforming growth-factor beta levels in rat wound chambers. *J. Surg. Res.* **42,** 622-628.

Cunningham M.E., Bergreen E.W., & McMillen M.A. (1996) Endothelin-stimulated monocytes and macrophages (M phi) produce neutrophil activating peptide-1 (NAP-1) and macrophage activating peptide-1 (MCP-1) in vitro. *FASEB J.* **10,** 1483.

Cunningham M.E., Huribal M., Bala R.J. *et al.* (1997) Endothelin-1 and endothelin-4 stimulate monocyte production of cytokines. *Crit. Care Med.* **25,** 958-964.

Danser A.H.J., Saris J.J., Schuijt M.P. & van Kats J.P. (1999) Is there a local renin-angiotensin system in the heart? *Cardiovasc. Res.* **44,** 252-265.

Darby I., Skalli O., & Gabbiani G. (1990) Alpha-smooth muscle actin is transiently expressed by myofibroblasts during experimental wound-healing. *Lab. Invest.* **63,** 21-29.

Dargie H.J. & McMurray J.J. (1994) Diagnosis and management of heart failure. *Br. Med. J.* **308,** 321-328.

Darzynkiewicz Z., Bruno S., Delbino G. *et al.* (1992) Features of apoptotic cells measured by flow cytometry. *Cytometry* **13,** 795-808.

Davenport A.P., O'Reilly G., Molenaar P., Maguire J., Kuc R.E., Sharkey A., Bacon C. & Ferro A. (1993) Human endothelin receptors characterised using reverse transcriptase-polymerase chain reaction, insitu hybridisation, and subtype-selective ligands BQ-123 and BQ-3020: evidence or expression of ET_B receptors in human vascular smooth muscle. *J.Cardiovasc.Pharmacol.* **22,** S22-S25.

Davenport A.P., Kuc R.E., Plumpton C., Mockridge J.W., Barker P.J. & Huskisson N.S. (1998) Endothelin-converting enzyme in human tissues. *Histochem. J.* **30,** 359-374.

Davis R.J. (1995) Transcriptional activation by MAP kinases. *Mol. Reprod. & Dev.* **42**, 459-467.

Dawes K.E., Cambrey A.D., Campa J.S. *et al.* (1996) Changes in collagen metabolism in response to endothelin-1: Evidence for fibroblast heterogeneity. *Int. J. Biochem. Cell. Biol.* **28,** 229-238.

De Lombaert S., Blanchard L., Stamford L.B. *et al.* (2000) Potent and selective non-peptide inhibitors of endothelin-converting enzyme-1 with sustained duration of action. *J. Med. Chem.* **43,** 488-504.

De Mey J.G. & Vanhoutte P.M. (1983) Anoxia and endothelium-dependent reactivity of the canine femoral artery. *J. Physiol.* **335,** 67-74.

De Mey J.G. & Moeremans M. (1986) Raising and testing polyclonal antibodies for immunohistochemistry. In: *Immunocytochemistry, Modern Methods and Applications.* (eds Polack J.M. & Van Noorden S.), pp. 3-12. Wright, Bristol.

Desmouliere A., Geinoz A., Gabbiani F. & Gabbiani G. (1993) Transforming growth factor beta-1 induces alpha-smooth muscle actin expression in granulation-tissue myofibroblasts and in quiescent and growing cultured fibroblasts. *J. Cell Biol.* **122,** 103-111.

Detar R. & Bohr D.F. (1972) Contractile responses of isolated vascular smooth muscle during prolonged exposure to anoxia. *Am.J.Physiol. (Heart Circ.Physiol)* **222,** 1269-1277.

Devesly P., Phillips P.E., Johns A., Rubanyi G. & Parker-Botelho L.H. (1990) Receptor kinetics differ for endothelin-1 and endothelin-2 binding to swiss 3T3 fibroblasts. *Biochem. Biophys. Res. Commun.* **172,** 126-134.

Dickstein K., Chang P., Willenheimer R. *et al.* (1995) Comparison of the effects of losartan and enalapril on clinical status and exercise performance in patients with moderate or severe chronic heart-failure. *J. Am. Coll. Cardiol.* **26,** 438-445.

Doggrell S.A. & Brown L. (1998) Rat models of hypertension, cardiac hypertrophy and failure. *Cardiovasc. Res.* **39,** 89-105.

Douglas S.A., James S. & Hiley C.R. (1991) Endothelial modulation and changes in endothelin pressor activity during hypoxia in the rat isolated perfused superior mesenteric arterial bed. *Br. J. Pharmacol.* **103,** 1441-1448.

Douglas S.A., Meek T.D. & Ohlstein E.H. (1994) Novel receptor antagonists welcome a new era in endothelin biology. *TIPS* **15,** 313-316.

Dupuis J., Goresky C.A. & Fournier A. (1996) Pulmonary clearance of circulating endothelin-1 in dogs in vivo: Exclusive role of ET(B) receptors. *J. App. Physiol.* **81,** 1510-1515.

Ehrenreich H., Anderson R.W., Fox C.H. *et al.* (1990) Endothelins, peptides with potent vasoactive properties, are produced by human macrophages. *J. Exper. Med.* **172,** 1741-1748.

Elshourbagy N.A., Lee J.A., Korman D.R. *et al.* (1992) Molecular-cloning and characterisation of the major endothelin receptor subtype in porcine cerebellum. *Mol. Pharmacol.* **41,** 465-473.

Elshourbagy N.A., Korman D.R., Wu H.L. *et al.* (1993) Molecular characterisation and regulation of the human endothelin receptors. *J. Biol. Chem.* **268,** 3873-3879.

Emori T., Hirata Y., Ohta K. *et al.* (1989) Concomitant secretion of big endothelin and its c-terminal fragment from human and bovine endothelial cells. *Biochem. Biophys. Res. Commun.* **162,** 217-223.

Emori T., Hirata Y. & Marumo F. (1990) Specific receptors for endothelin-3 in cultured bovine endothelial cells and its cellular mechanism of action. *Febs Lett.* **263,** 261-264.

Emori T., Hirata Y., Ohta K. *et al.* (1991) Cellular mechanism of endothelin-1 release by angiotensin and vasopressin. *Hypertension* **18,** 165-170.

Emoto N. & Yanagisawa M. (1995) Endothelin-converting enzyme-2 is a membrane-bound, phosphoramidon-sensitive metalloprotease with acidic pH optimum. *J. Biol. Chem.* **270,** 15262-15268.

Entman M.L., Youker K., Shappell S.B., Siegel C., Rothlein R., Dreyer W.J., Schmalstieg F.C. & Smith C.W. (1990) Neutrophil adherence to isolated adult canine myocytes - evidence for a cd18-dependent mechanism. *J. Clin. Invest.* **85,** 1497-1506.

Ercan Z.S., Ilhan M., Kilinc M. & Turker R.K. (1996) Arrhythmogenic action of endothelin peptides in isolated perfused whole hearts from guinea pigs and rats. *Pharmacology* **53,** 234-240.

Evans H.G., Lewis M.J. & Shah A.M. (1994) Modulation of myocardial relaxation by basal release of endothelin from endocardial endothelium. *Cardiovasc. Res.* **28,** 1694-1699.

Fajardo L.F., Prionas S.D., Kwan H.H. *et al.* (1996) Transforming growth factor beta 1 induces angiogenesis *in vivo* with a threshold pattern. *Lab. Invest.* **74,** 600-608.

Falk E. & Shah P.K. (1996) Pathology of acute ischaemic syndromes. In: *Acute myocardial infarction and other acute ischaemic syndromes.* Ch.3, Edition 8, ed Califf RM, Mosby.

Fareh J., Touyz R.M., Schiffrin E.L. & Thibault G. (1996) Endothelin-1 and angiotensin II receptors in cells from rat hypertrophied heart - Receptor regulation and intracellular Ca^{2+} modulation. *Circ. Res.* **78,** 302-311.

Ferrari R. & Agnoletti G. (1989) Atrial natriuretic peptide - its mechanism of release from the atrium. *Int. J. cardiol.* **24,** 137-149.

Ferrari R., Ceconi C., Curello S. & Visioli O. (1998) The neuroendocrine and sympathetic nervous system in congestive heart failure. *Eur. Heart J.* **19,** F45-F51.

Filep J.G., Fournier A., & Foldesfilep E. (1995) Acute pro-inflammatory actions of endothelin-1 in the guinea-pig lung - involvement of ET(A) And ET(B) receptors. *Br. J. Pharmacol.* **115,** 227-236.

Fishbein M.C., Maclean D. & Maroko P.R. (1978) The histopathological evolution of myocardial infarction. *Chest* **73,** 843-849.

Force T. (1998) Mechanisms of endothelin-induced mitogenesis in vascular smooth muscle cells. In: *Endothelin: Molecular Biology, Physiology, and Pathology.*, pp. 121-166. Human Press.

Force T. & Bonventre J.V. (1998) Growth factors and mitogen activated protein kinases. *Hypertension* **31,** 152-161.

Forssmann W.G., Richter R. & Meyer M. (1998) The endocrine heart and natriuretic peptides: histochemistry, cell biology, and functional aspects of the renal urodilatin system. *Histochem. & Cell Biol.* **110,** 335-357.

Fraccarollo D., Hu K., Galuppo P. *et al.* (1997) Chronic endothelin receptor blockade attenuates progressive ventricular dilatation and improves cardiac function in rats with myocardial infarction. *Circulation* **96**, 3963-3973.

Frangogiannis N.G., Youker K.A., Rossen R.D. *et al.* (1998) Cytokines and the microcirculation in ischemia and reperfusion. *J. Mol. Cell. Cardiol.* **30,** 2567-2576.

Fukuroda T., Noguchi K., Tsuchida S. *et al.* (1990) Inhibition of biological actions of big endothelin-1 by phosphoramidon. *Biochem. Biophys. Res. Commun.* **172,** 390-395.

Fukuroda T., Fujikawa T., Ozaki S. *et al.* (1994) Clearance of circulating endothelin-1 by ET(B) receptors in rats. *Biochem. Biophys. Res. Commun.* **199,** 1461-1465.

Gabriel A., Kuddus R.H., Rao A.S. *et al.* (1999) Down-regulation of endothelin receptors by transforming growth factor beta 1 in hepatic stellate cells. *Journal of Hepatology* **30,** 440-450.

Gall J.G. & Pardue M. (1969) Formation and detection of RNA-DNA hybrid molecules in cytological preparations. *Proc. Natl. Acad. Sci.* **63,** 378-383.

Galuppo P., Fraccarollo D., Gaudron P. *et al.* (1999) Endothelin A receptor blockade after myocardial infarction promotes early infarct expansion. *Circulation* **100,** 2963.

Ganten D., Lindpaintner K., Ganten U. *et al.* (1991) Transgenic rats -new animal models in hypertension research. *Hypertension* **17,** 843-855.

Garjani A., Wainwright C.L., Zeitlin I.J., Wilson C. & Slee S.J. (1995) Effects of endothelin-1 and the ET(A)-Receptor antagonist, BQ-123, on ischaemic arrhythmias in anaesthetised rats. *J. Cardiovasc. Pharmacol.* **25,** 634-642.

Gavrieli Y., Sherman Y. & Ben-Sasson S.A. (1992) Identification of programmed cell death *in situ* via specific labelling of nuclear DNA fragmentation. *J. Cell. Biol.* **119,** 493-501.

Geshi E., Nomizo A., Arata Y., *et al.* (1999) Effect of non-selective endothelin blockade, TAK-044, on the ischaemic cellular injury of rat heart. *Basic Res. Cardiol.* **94,** 94-101.

Giaid A., Kimura S., Chen M.F. *et al.* (1991) Immunocytochemical localisation of endothelin-like immunoreactivity in the heart of man and rat. *Hypertension* **17,** 444-445.

Giaid A., Saleh D., Yanagisawa M. & Forbes R.D.C. (1995) Endothelin-1 immunoreactivity and messenger-RNA in the transplanted human heart. *Transplantation* **59,** 1308-1313.

Gillespie M., Owasayo J., McMurty I. & O'Brien R. (1986) Sustained coronary vasoconctriction provoked by a peptidergic substance released from endothelial cells in culture. *Jn. Pharmacol. Exp. Ther.* **236,** 339-343.

Golden C.L., Nick H.S. & Visner G.A. (1998) Thrombin regulation of endothelin-1 gene in isolated human pulmonary endothelial cells. *Am. J. Physiol.-Lung Cell.r & Mol. Physiol.* **18,** L854-L863.

Goligorsky M.S., Budzikowski A.S., Tsukahara H. *et al.* (1999) Co-operation between endothelin and nitric oxide in promoting endothelial cell migration and angiogenesis. *Clin. Exp. Pharmacol. Physiol.* **26,** 269-271.

Gonon A.T., Wang Q.D., Shimizu M. *et al.* (1998) The novel non-peptide selective endothelin A receptor antagonist LU 135252 protects against myocardial ischaemic and reperfusion injury in the pig. *Acta Physiol. Scand.* **16,** 131-137.

Gonzalez F., Seth A., Raden D., Bowman D., Fay F. & Davis R. (1993) Serum-induced translocation of mitogen-activated protein kinase to the cell surface ruffling membrane and the nucleus. *J. Cell Biol.* **122,** 1089-1101.

Goodwin A.T., Amrani M., Gray G.C., Jayakumar J. & Yacoub M. (1998) Role of endogenous endothelin in the regulation of basal coronary tone in the rat. *J. Physiol.* **511,** 549-557.

Goto K. & Warner T.D. (1995) Molecular pharmacology -endothelin versatility. *Nature* **375,** 539-540.

Gray G.A., Breu V. & Clozel M. (1995) Endothelin receptors that modulate contraction of the rat fundus. *J. Cardiovasc. Pharmacol.* **26,** S126-S129.

Gray G.A. & Webb D.J. (1996) The endothelin system and its potential as a therapeutic target in cardiovascular disease. *Pharmacol.Ther.* **72,** 109-148.

Gray G.A., Mickley E.J., Webb D.J. & McEwan P.E. (2000) Localisation and function of ET-1 and ET receptors in small arteries post-myocardial infarction: Upregulation of smooth muscle ET_B receptors that modulate contraction. *Br. J. Pharmacol.* **130,** 1735-1744.

Gray M.O., Long C.S., Kalinyak J.E. *et al.* (1998) Angiotensin II stimulates cardiac myocyte hypertrophy via paracrine release of TGF-beta(1) and endothelin-1 from fibroblasts. *Cardiovasc. Res.* **40,** 352-363.

Gronostajski R.M. (1987) Site-specific DNA-binding of nuclear factor-i - effect of the spacer region. *Nucleic Acids Res.* **15,** 5545-5559.

Grover G.J., Dzwonczyk S. & Parham C.S. (1993) The endothelin-1 receptor antagonist BQ-123 reduces infarct size in a canine model of coronary-occlusion and reperfusion. *Cardiovasc. Res.* **27,** 1613-1618.

Guarda E., Katwa L.C. & Weber K.T. (1993) Effects of endothelin on collagen turnover in cardiac fibroblasts. *Cardiovasc. Res.* **27,** 2130-2134.

Gui G., Xu D., Emoto N. & Yanagasawia M. (1993) Intracellular localisation of membrane-bound endothelin-converting enzyme from rat lung. *J. Cardiovasc. Pharmacol.* **22,** S35-S56.

Hafizi S., Goodwin A.T., Chester A.H. & Yacoub M.H. (1996) Endothelin-1 is a potent co-mitogen for cultured human coronary artery smooth muscle cells: Synergy with growth factors. *Circulation* **94,** 1391-1391.

Hafizi S., Wharton J., Chester A.H. *et al.* (1998) Endothelin-1 stimulates collagen synthesis in cultured human cardiac fibroblasts via the ET_A receptor. *Circulation* **98,** 922.

Hafizi S., Allen S.P., Goodwin A.T., Chester A.H. & Yacoub M.H. (1999) Endothelin-1 stimulates proliferation of human coronary smooth muscle cells via the ET_A receptor and is co-mitogenic with growth factors. *Atherosclerosis* **146,** 351-359.

Hagar J.M. (1994) Endogenous endothelin-1 impairs Endothelium-dependent relaxation after myocardial-ischemia and reperfusion. *Am. J. Physiol. (Heart Circ. Physiol.)* **36,** H1833-H1841.

Hand M.F., Haynes W.G. & Webb D.J. (1999) Reduced endogenous endothelin-1-mediated vascular tone in chronic renal failure. *Kid. Int.* **55,** 613-620.

Hanehira T., Kohno M. & Yoshikawa J. (1997) Endothelin production in cultured vascular smooth muscle cells - Modulation by the atrial, brain, and C-type natriuretic peptide system. *Metab.-Clin. & Exp.* **46,** 487-493.

Hansen C.A., Schroering A.G. & Robishaw J.D. (1995) Subunit expression of signal-transducing G-proteins in cardiac tissue -implications for phospholipase C-beta regulation. *J. Mol. Cell. Cardiol.* **27,** 471-484.

Harrison V.J., Barnes K., Turner A.J. *et al.* (1995) Identification of endothelin-1 and big endothelin-1 in secretory vesicles isolated from bovine aortic endothelial cells. *Proc. Natl. Acad. Sci.* **92,** 6344-6348.

Haynes W.G. & Webb D.J. (1994) Contribution of endogenous generation of endothelin-1 to basal vascular tone. *Lancet* **344,** 852-854.

Haynes W.G., Strachan F.E., Gray G.A. & Webb D.J. (1995a) Forearm vasoconstriction to endothelin-1 is mediated by ET(A) and ET(B) receptors *in vivo* in humans. *J. Cardiovasc. Pharmacol.* **26,** S40-S43.

Haynes W.G., Strachan F.E. & Webb D.J. (1995b) Endothelin ET(A) and ET(B) receptors cause vasoconstriction of human resistance and capacitance vessels *in vivo*. *Circulation* **92,** 357-363.

Haynes W.G., Ferro C.J., Okane K.P.J. *et al.* (1996) Systemic endothelin receptor blockade decreases peripheral vascular resistance and blood pressure in humans. *Circulation* **93,** 1860-1870.

Haywood G.A., Gullestad L., Katsuya T. *et al.* (1997) AT(1) and AT(2) angiotensin receptor gene expression in human heart failure. *Circulation* **95,** 1201-1206.

Herizi A., Jover B., Bouriquet N. & Mimran A. (1998) Prevention of the cardiovascular and renal effects of angiotensin II by endothelin blockade. *Hypertension* **31,** 10-14.

Hide E.J., Piper J. & Thiemermann C. (1995) Endothelin-1-induced reduction of myocardial infarct size by activation of ATP-sensitive potassium channels in a rabbit model of myocardial-ischaemia and reperfusion. *Br. J. Pharmacol.* **116,** 2597-2602.

Hilaldandan R., Urasawa K. & Brunton L.L. (1992) Endothelin inhibits adenylate-cyclase and stimulates phosphoinositide hydrolysis in adult cardiac myocytes. *J. Biol. Chem.* **267,** 10620-10624.

Hillis G.S., Mangione A., Dalsey W. *et al.* (1999) Neutrophil CD18 adhesion molecule expression predicts infarct size in a rabbit model of myocardial infarction. *Circulation* **100,** 4284.

Hinson J.P., Kapas S., Teja R. & Vinson G.P. (1991) Effect of the endothelins on aldosterone secretion by rat zona glomerulosa cells *in vitro*. *J. Ster. Biochem. Mol. Biol.* **40,** 437-439.

Hirata Y., Yoshimi H., Takaichi S., Yanagisawa M. & Masaki T. (1988) Binding and receptor downregulation of a novel vasoconstrictor endothelin in cultured rat vascular smooth muscle cells. *Febs Lett.* **239,** 13-17.

Hirata Y., Fukuda Y., Yoshimi H. *et al.* (1989) Specific receptor for endothelin in cultured rat cardiocytes. *Biochem. Biophys. Res. Commun.* **160,** 1438-1444.

Hirata Y., Kanno K., Watanabe T.X. *et al.* (1990) Receptor binding and vasoconstrictor activity of big endothelin. *Eur. J. Pharmacol.* **176,** 225-228.

Hirsch A.T., Talsness C.E., Schunkert H., Paul M. & Dzau V.J. (1991) Tissue-specific activation of cardiac angiotensin converting enzyme in experimental heart failure. *Circ. Res.* **69,** 475-482.

Hokimoto S., Yasue H., Fujimoto K. *et al.* (1996) Expression of angiotensin-converting enzyme in remaining viable myocytes of human ventricles after myocardial infarction. *Circulation* **94,** 1513-1518.

Hood W.B., McCarthy B. & Lown B. (1967) Myocardial infarction following coronary ligation in dogs: haemodynamic effects of isoprotorenol and acetylstrophantidin. *Circ. Res.* **21,** 199.

Hori S., Komatsu Y., Shigemoto R., Mizuno N. & Nakanishi S. (1992) Distinct tissue distribution and cellular-localisation of 2 messenger ribonucleic-acids encoding different subtypes of rat endothelin receptors. *Endocrinology* **130,** 1885-1895.

Hosoda K., Nakao K., Tamura N. *et al.* (1992) Organisation, structure, chromosomal assignment, and expression of the gene encoding the human endothelin-a receptor. *J. Biol. Chem.* **267,** 18797-18804.

Hosoda K., Hammer R.E., Richardson J.A. *et al.* (1994) Targeted and natural (Piebald-lethal) Mutations of endothelin-b receptor gene produce megacolon associated with spotted coat colour in mice. *Cell* **79,** 1267-1276.

Hsu S.M., Raine L. & Fanger H. (1981) Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabelled antibody (PAP) procedures. *J. Histochem. Cytochem.* **29,** 577-580.

Hu J., Discher D.J., Bishopric N.H. & Webster K.A. (1998) Hypoxia regulates expression of the endothelin-1 gene through a proximal hypoxia-inducible factor-1 binding site on the antisense strand. *Biochem. Biophys. Res. Commun.* **245,** 894-899.

Hu K., Gaudron P., Schmidt T.J., *et al.* (1998) Aggravation of left ventricular remodelling by a novel specific endothelin ET_A antagonist EMD94246 in rats with experimental myocardial infarction. *J. Cardiovasc. Pharmacol.* **32,** 505-508.

Hupf H., Grimm D., Riegger G.A.J. & Schunkert H. (1999) Evidence for a vasopressin system in the rat heart. *Circ. Res.* **84,** 365-370.

Hutchinson I.V. (1998) An endothelin-transforming growth factor beta pathway in the nephrotoxicity of immunosuppressive drugs. *Curr. Opin. Nephrol. Hypert.* **7,** 665-671.

Ichikawa K., Hidai C., Okuda C. & Kimata S. (1996) Endogenous endothelin-1 modulates cardiac hypertrophy and switching of myosin heavy chain gene expression in rat ventricular myocardium. *J. Am. Coll. Cardiol.* **27,** 1286-1291.

Ignarro L.J. (1989) Endothelium-derived nitric oxide -actions and properties. *FASEB J.* **3,** 31-36.

Ikura T., Sawamura T., Shiraki T. *et al.* (1994) cDNA cloning and expression of bovine endothelin-converting enzyme. *Biochem. Biophys. Res. Commun.* **203,** 1417-1422.

Imai T., Hirata Y., Eguchi S. *et al.* (1992) Concomitant expression of receptor subtype and isopeptide of endothelin by human adrenal-gland. *Biochem. Biophys. Res. Commun.* **182,** 1115-1121.

Inoue A., Yanagisawa M., Kimura S. *et al.* (1989) The human endothelin family - 3 structurally and pharmacologically distinct isopeptides predicted by 3 separate genes. *Proc. Natl. Acad. Sci.* **86,** 2863-2867.

Ishikawa T., Yanagisawa M., Kimura S., Goto K. & Masaki T. (1988) Positive inotropic action of novel vasoconstrictor peptide endothelin on guinea-pig atria. *Am. J. Physiol.* **255,** H970-H973.

Ito H., Hirata Y. & Hiroe M. (1991) Endothelin-1 induces hypertrophy with enhanced expression of muscle-specific genes in cultured neonatal rat cardiomyocytes. *Circ. Res.* **69,** 209-215.

Itoh H., Pratt R.E. & Dzau V.J. (1990) Atrial natriuretic polypeptide inhibits hypertrophy of vascular smooth muscle cells. *J. Clin. Invest.* **86,** 1690-1697.

Iverson O.H. (1989) The cell kinetics of the inflammatory reaction. Introduction and overview. *Curr. Top. Path.* **79,** 1-5.

James A.F., Xie L.H., Fujitani Y., Hayashi S. & Horie M. (1994) Inhibition of the cardiac protein-kinase A-dependent chloride conductance by endothelin-1. *Nature* **370,** 297-300.

John H.A. & Birnstiel M.L. (1969) RNA-DNA hybrids at the cytological level. *Nature* **223,** 582-587.

Jordan J.E., Zhao Z.Q. & Vinten-Johansen J. (1999) The role of neutrophils in myocardial ischemia-reperfusion injury. *Cardiovasc. Res.* **43,** 860-878.

Kaddoura S. & PooleWilson P.A. (1996) Endothelin-1 in heart failure: A new therapeutic target? *Lancet* **348,** 418-419.

Kajstura J., Cheng W., Reiss K. *et al.* (1996) Apoptotic and necrotic myocyte cell deaths are independent contributing variables of infarct size in rats. *Laboratory Investigation* **74,** 86-107.

Kanno K., Hirata Y., Tsujino M. *et al.* (1993) Up-regulation of ET_B receptor subtype mRNA by angiotensin II in rat cardiomyocytes. *Biochem. Biophys. Res. Commun.* **194,** 1282-1286.

Kanse S.M., Takahashi K., Lam H.C. *et al.* (1991) Cytokine stimulated endothelin release from endothelial cells. *Life Sci.* **48,** 1379-1384.

Karaki H., Sudjarwo A., Hori M. *et al.* (1993) Induction of Endothelium-dependent relaxation in the rat aorta by IRL-1620, a novel and selective agonist at the endothelin ET(B)- Receptor. *Br. J. Pharmacol.* **109,** 486-490.

Karne S., Jayawickreme C.K. & Lerner M.R. (1993) Cloning and characterisation of an endothelin-3 specific receptor ET(C) Receptor From xenopus-laevis dermal melanophores. *J. Biol. Chem.* **268,** 19126-19133.

Katwa L.C., Guarda E. & Weber K.T. (1993) Endothelin receptors in cultured adult-rat cardiac fibroblasts. *Cardiovasc. Res.* **27,** 2125-2129.

Katwa L.C., Campbell S.E., Tanner M.A. & Myers P.R. (1999) The upregulation of endothelin and its receptors in porcine coronary arteries in a double balloon injury model of restenosis. *Basic Res. Cardiol.* **94,** 445-453.

Kelso E.J., McDermott B.J., Silke B. & Spiers J.P. (1996) Contractile effects of endothelin-1 and endothelin-3, mediated by ET(A) and ET(B) receptor subtypes, in ventricular cardiomyocytes isolated from rabbit myocardium. *Br. J. Pharmacol.* **118,** 131.

Kelso E.J., McDermott B.J., Silke B. & Spiers J.P. (2000) Endothelin A receptor subtype mediates endothelin-induced contractility in left ventricular cardiomyocytes isolated from rabbit myocardium. *J. Pharmacol. Exp. Ther.* **294,** 1047-1052.

Khan M.A., Dashwood M.R., Mumtaz F.H. *et al.* (1999) Upregulation of endothelin a receptor sites in the rabbit diabetic kidney: Potential relevance to the early pathogenesis of diabetic nephropathy. *Nephron* **83,** 261-267.

Kim S. & Iwao H. (2000) Molecular and cellular mechanisms of angiotensin II-mediated cardiovascular and renal diseases. *Pharmacol. Rev.* **52,** 11-34.

King J.M., Srivastava K.D., Stefano G.B. *et al.* (1997) Human monocyte adhesion is modulated by endothelin B receptor-coupled nitric oxide release. *J. Immunol.* **158,** 880-886.

King K.L., Winer J. & Mather J.P. (1996) Endogenous cardiac vasoactive factors modulate endothelin production by cardiac fibroblasts in culture. *Endocrine* **5,** 95-102.

Kiowski W., Sutsch G., Hunziker P. *et al.* (1995) Evidence for endothelin-1-mediated vasoconstriction in severe chronic heart failure. *Lancet* **346**, 732-736.

Kirschenlohr H.L., Metcalfe J.C., Weissberg P.L. *et al.* (1993) Adult human aortic smooth muscle cells in culture produce active TGF- beta. *Am. J. Physiol.* **265,** C571-C576.

Kloog Y., Ambar I., Sokolovsky M. *et al.* (1988) Sarafotoxin, a novel vasoconstrictor peptide -phosphoinositide hydrolysis in rat-heart and brain. *Science* **242,** 268-270.

Kobayashi T., Miyauchi T., Sakai S. *et al.* (1999) Expression of endothelin-1, ET_A and ET_B receptors, and ECE and distribution of endothelin-1 in failing rat heart. *Am. J. Physiol. (Heart Circ. Physiol.)* **45,** H1197-H1206.

Kohan D.E. (1997) Endothelins in the normal and diseased kidney. *American Journal of Kidney Diseases* **29,** 2-26.

Kojda G., Kottenberg K., Nix P. *et al.* (1996) Low increase in cGMP induced by organic nitrates and nitro-vasodilators improves contractile response of rat ventricular myocytes. *Circ. Res.* **78,** 91-101.

Kojima M., Kusumoto K., Fujiwara S. *et al.* (1995) Role of endogenous endothelin in the extension of myocardial infarct size studied with the endothelin receptor antagonist, TAK-044. *J. Cardiovasc. Pharmacol.* **26,** S365-S368.

Kourembanas S., Marsden P.A., Mcquillan L.P. *et al.* (1991) Hypoxia induces endothelin gene-expression and secretion in cultured human endothelium. *J. Clin. Invest.* **88,** 1054-1057.

Kourembanas S., Morita T., Liu Y.X. & Christou H. (1997) Mechanisms by which oxygen regulates gene expression and cell-cell interaction in the vasculature. *Kid. Int.* **51,** 438-443.

Kulkarni A.B., Huh C.G., Becker D. *et al.* (1993) Transforming growth factor-beta-1 null mutation in mice causes excessive inflammatory response and early death. *Proc. Natl. Acad. Sci.* **90,** 770-774.

Kumar C., Mwangi V., Nuthulaganti P. *et al.* (1994) Cloning and characterisation of a novel endothelin receptor from xenopus heart. *J. Biol. Chem.* **269,** 13414-13420.

Kurihara H., Yoshizumi M., Sugiyama T. *et al.* (1989) Transforming growth factor-beta stimulates the expression of endothelin messenger-RNA by vascular endothelial cells. *Biochem. Biophys. Res. Commun.* **159,** 1435-1440.

Kurihara Y., Kurihara H., Suzuki H. *et al.* (1994) Elevated blood pressure and craniofacial abnormalities in mice deficient in endothelin-1. *Nature* **368,** 703-710.

Kusumoto K., Fujiwara A., Ikeda S., Watanabe T. & Fujino M. (1996) Pharmacological characterisation of cardiovascular responses induced by endothelin-1 in the perfused rat heart. *Eur. J. Pharmacol.* **296,** 65-74.

Kuwaki T., Cao W.H. & Kumada M. (1994) Endothelin in the brain and its effect on central control of the circulation and other functions. *Jap. J. Physiol.* **44,** 1-18.

Kuwaki T., Kurihara Y., Kurihara H., Yazaki Y. & Kumada M. (1995) Role of endothelin in central cardiorespiratory control - modern and classical approaches. *News in Physiol. Sci.* **10,** 228-233.

Krause S.M., Lynch J.J., Stabilito I.I., & Woltmann R.F. (1994) Intravenous administration of the endothelin-1 antagonist BQ-123 does not ameliorate myocardial ischaemic injury following acute coronary artery occlusion in the dog. *Cardiovasc. Res.* **28,** 1672-1678.

Krum H., Charlon V., Wildmann T., & Packer M. (1999) Long-term open-label experience with an endothelin receptor antagonist, bosentan, in patients with severe chronic heart failure. *Circulation* **100,** A3408.

Kuizinga M.C., Smits J.F.M., Arends J.W. *et al.* (1998) AT(2) receptor blockade reduces cardiac interstitial cell DNA synthesis and cardiac function after rat myocardial infarction. *J. Mol. Cell. Cardiol.* **30,** 425-434.

Kurihara H., Yoshizumi M., Sugiyama T. *et al.* (1989) Transforming growth factor-beta stimulates the expression of endothelin messenger-RNA by vascular endothelial cells. *Biochem. Biophys. Res. Commun.* **159,** 1435-1440.

Lal H., Williams K.I. & Woodward B. (1998) Comparative study of effects of ET-1, big ET-1 and SX6C in rat isolated pulmonary arteries and veins. *Br. J. Pharmacol.* **123,** 291.

Lang R.M., Elkayam U., Yellen L.G. *et al.* (1997) Comparative effects of losartan and enalapril on exercise capacity and clinical status in patients with heart failure. *J. Am. Coll. Cardiol.* **30,** 983-991.

La Villa G., Lazzeri C., Fronzaroli C., Franchi F. & Gentilini P. (1995) Brain natriuretic peptide. *Ann. Ital. Med. Int.* **10,** 233-241.

Lechat P., Packer M., Chalon S. *et al.* (1998) Clinical effects of beta-adrenergic blockade in chronic heart failure - A meta-analysis of double-blind, placebo-controlled, randomised trials. *Circulation* **98,** 1184-1191.

Lechat P., Brunhuber K.W., Hofmann R., *et al.* (1999) The Cardiac Insufficiency Bisoprolol Study II (CIBIS-II): a randomised trial. *Lancet* **353,** 9-13.

Lechleitner P. (1993) Endothelin-1 in patients with complicated and uncomplicated myocardial infarction. *Clin. Invest.* **70,** 1070-1072.

Lee A.A., Dillmann W.H., Mcculloch A.D. *et al.* (1995) Angiotensin-II stimulates the autocrine production of transforming growth factor-beta-1 in adult rat cardiac fibroblasts. *J. Mol. Cell. Cardiol.* **27,** 2347-2357.

Lee J.Y., Warner R.B., Adler A.L. *et al.* (1994) The ET_A receptor antagonist FR139317 reduces myocardial infarction induced by coronary artery occlusion and reperfusion in the rat. *FASEB J.* **8,** A854.

Lenormand P., Sardet C. *et al.* (1993) Growth factors induce nuclear translocation of MAP kinases (P42(Mapk) And p44(Mapk)) but not of their activator MAP kinase kinase (P45(Mapkk)) in fibroblasts. *J. Cell Biol.* **122,** 1079-1088.

Li H.B., Chen S.J., Chen Y.F. *et al.* (1994) Enhanced endothelin-1 and endothelin receptor gene expression in chronic hypoxia. *J. App. Physiol.* **77,** 1451-1459.

Li J.M. & Brooks G. (2000) Differential protein expression and subcellular distribution of TGF beta-1, beta-2 and beta-3 in cardiomyocytes during pressure overload-induced hypertrophy. *J. Mol. Cell. Cardiol.* **29,** 2213-2224.

Lijnen P. & Petrov V. (1999) Renin-angiotensin system, hypertrophy and gene expression in cardiac myocytes. *J. Mol. Cell. Cardiol.* **31,** 949-970.

Lin H.Y., Kaji E.H., Winkel G.K., Ives H.E. & Lodish H.F. (1991) Cloning and functional expression of a vascular smooth muscle endothelin-1 receptor. *Proc. Natl. Acad. Sci.* **88,** 3185-3189.

Lindpaintner K., Lu W.Y., Niedermajer N. *et al.* (1993) Selective activation of cardiac angiotensinogen gene expression in post-infarction ventricular remodelling in the rat. *J. Mol. Cell. Cardiol.* **25,** 133-143.

Ling B.N. (1994) Luminal endothelin-1 inhibits apical Na and Cl channels in cultured rabbit CCT principal cells. *J. Am. Soc. Neph.* **5,** 292.

Liu Y.H., Yang X.P., Sharov V.G. *et al.* (1997) Effects of angiotensin-converting enzyme inhibitors and angiotensin II type 1 receptor antagonists in rats with heart failure - Role of kinins and angiotensin II type 2 receptors. *J. Clin. Invest.* **99,** 1926-1935.

Loffler B.M., Kalina B. & Kunze H. (1991) Partial characterisation and subcellular-distribution patterns of endothelin-1, endothelin-2 and endothelin-3 binding sites in human liver. *Biochem. Biophys. Res. Commun.* **181,** 840-845.

Lonn E. & McKelvie R. (2000) Regular review - Drug treatment in heart failure. *Br. Med. J.* **320,** 1188-1192.

Love M.P., Haynes W.G., Webb D.J. & McMurray J.J.V. (1994) Anti-endothelin therapy is of potential benefit in heart failure. *Circulation* **90,** 547.

MacCarthy P.A., Grocott-Mason R., Prendergast B.D. & Shah A.M. (2000) Contrasting inotropic effects of endogenous endothelin in the normal and failing human heart - Studies with an intra-coronary ET_A receptor antagonist. *Circulation* **101,** 142-147.

Macchi C., Malendowicz L.K., Meneghelli V. & Nussdorfer G.G. (1999) Endothelin-1 stimulates arginine-vasopressin and atrial natriuretic peptide release by rat adrenal medulla: receptor subtypes involved and possible functional relevance. *Med. Sci. Res.* **27,** 163-165.

Machado R.D.P., Santos R.A.S., & Andrade S.P. (1999) Opposing actions of angiotensins on angiogenesis. *Life Sci.* **66,** 67-76.

MacKenna B.R. & Calander R. (1997) Transport Sysytems: The heart, blood vessels and body fluids. In: *Illustrated Physiology*, 6th edn, pp.102-108. Churchill Livingstone.

MacLellan W.R. & Schneider M.D. (1997) Death by design - Programmed cell death in cardiovascular biology and disease. *Circ. Res.* **81,** 137-144.

MacMahon S., Sharpe N., Doughty R., *et al.* (1997) Randomised, placebo-controlled trial of carvedilol in patients with congestive heart failure due to ischaemic heart disease. *Lancet* **349,** 375-380.

Maguire J.J., Kuc R.E., Oreilly G. & Davenport A.P. (1994) Vasoconstrictor endothelin receptors characterised in human renal artery and vein *in vitro*. *Br. J. Pharmacol.* **113,** 49-54.

Malatino L.S., Grassi R., Stancanelli B. *et al.* (1993) Release of immunoreactive endothelin from the heart during percutaneous transluminal coronary angioplasty. *Am. Heart. J.* **126,** 700-702.

Malek A.M., Zhang J., Jiang J.W., Alper S.L. & Izumo S. (1999) Endothelin-1 gene suppression by shear stress: Pharmacological evaluation of the role of tyrosine kinase, intracellular calcium, cytoskeleton, and mechanosensitive channels. *J. Mol. Cell. Cardiol.* **31,** 387-399.

Margulies K.B., Hildebrand F.L., Lerman A., Perrella M.A. & Burnett J.C. (1990) Increased endothelin in experimental heart failure. *Circulation* **82,** 2226-2230.

Maroko P.R., Kjekshus J.K. & Sobel B.E. (1971) Factors influencing infarct size following experimental coronary artery occlusions. *Circulation* **67,** 1016-1023.

Marx N., Neumann F.J., Ott I. *et al.* (1997) Induction of cytokine expression in leukocytes in acute myocardial infarction. *J. Am. Coll. Cardiol.* **30,** 165-170.

Massart P.E., Hodeige D.G., Van Mechelen H. *et al.* (1998) Angiotensin II and endothelin-1 receptor antagonists have cumulative hypotensive effects in canine Page hypertension. *J. Hypertens.* **16,** 835-841.

Mathison Y. & Israel A. (1998) Endothelin ET_B receptor subtype mediates nitric oxide cGMP formation in rat adrenal medulla. *Brain Res. Bull.* **45,** 15-19.

Matsumoto T., Wada A., Tsutamoto T. *et al.* (1999) Vasorelaxing effects of atrial and brain natriuretic peptides on coronary circulation in heart failure. *Am. J. Physiol. (Heart Circ. Physiol.)* **45,** H1935-H1942.

Matsumura Y., Hisaki K., Takaoka M. & Morimoto S. (1990) Phosphoramidon, a metalloprotease inhibitor, suppresses the hypertensive effect of big endothelin-1. *Eur. J. Pharmacol* **185,** 103-106.

Maxwell M.J., Goldie R.G. & Henry P.J. (1998) Ca^{2+} signalling by endothelin receptors in rat and human cultured airway smooth muscle cells. *Br. J. Pharmacol.* **125,** 1768-1778.

Maxwell M.P., Hearse D.J. & Yellon D.M. (1987) Species variation in the coronary circulation during regional myocardial ischaemia: a critical determinant of the rate of evolution and extent of myocardial infarction. *Cardiovasc. Res.* **21,** 737-746.

McDonald D., Bailie J., Archer D. & Chakravarthy U. (1995) Molecular characterisation of endothelin receptors and the effect of insulin on their expression in retinal microvascular pericytes. *J. Cardiovasc. Pharmacol.* **26,** S287-S289.

McEwan P.E., Valdenaire O., Sutherland L., Webb D.J. & Gray G.A. (1998a) A non-radioactive method for localisation of endothelin receptor mRNA *in situ*. *J. Cardiovasc. Pharmacol.* **31,** S443-S446.

McEwan P.E., Sherry L., Webb D.J. & Gray G.A. (1998b) Localisation of myocardial prepro endothelin-1 mRNA and endothelin-1 in a rat model of chronic heart failure. *J. Mol. Cell. Cardiol.* **30**, A101.

McEwan P.E., Sherry L., Kenyon C.J., Webb D.J. & Gray G.A. (2000) Regulation of the myocardial endothelin system by angiotensin II and losartan. *J. Cardiovasc. Pharmacol.* in press.

McKay R.G., Pfeffer M.A., Pasternak R.C. *et al.* (1986) Left-ventricular remodelling after myocardial infarction - a corollary to infarct expansion. *Circulation* **74,** 693-702.

McMillen M.A., Huribal M., Cunningham M.E. *et al.* (1995) Endothelin-1 increases intracellular calcium in human monocytes and causes production of interleukin-6. *Crit. Care Med.* **23,** 34-40.

McMurdo L., Corder R., Thiemermann C. & Vane J.R. (1993) Incomplete inhibition of the pressor effects of endothelin-1 and related peptides in the anaesthetised rat with BQ-123 provides evidence for more than one vasoconstrictor receptor. *Br. J. Pharmacol.* **108,** 557-561.

McMurray J.J., Ray S.G., Abdullah I., Dargie H.J. & Morton J.J. (1992) Plasma endothelin in chronic heart failure. *Circulation* **85,** 1374-1379.

Meyer M., Lehnart S., Pieske B. *et al.* (1996) Influence of endothelin 1 on human atrial myocardium - Myocardial function and subcellular pathways. *Basic Res. Cardiol.* **91,** 86-93.

Michel J.B., Lattion A.L., Salzmann J.L. *et al.* (1988) Hormonal and cardiac effects of converting enzyme inhibition in rat myocardial infarction. *Circ. Res.* **62,** 641-650.

Michel J.B., Nicolletti A. & Arnal J.F. (1995) Left ventricular remodelling following experimental myocardial infarction. *Eur. Heart J.* **16,** 49-57.

Mickley E.J., Gray G.A. & Webb D.J. (1997) Activation of endothelin ET(A) receptors masks the constrictor role of endothelin ET(B) receptors in rat isolated small mesenteric arteries. *Br. J. Pharmacol.* **120,** 1376-1382.

Minshall R.D., Nakamura F., Becker R.P. & Rabito S.F. (1995) Characterisation of bradykinin B2 receptors in adult myocardium and neonatal rat cardiomyocytes. *Circ. Res.* **76,** 773-780.

Miyauchi T., Yanagisawa M., Tomizawa T. *et al.* (1989) Increased plasma-concentrations of endothelin-1 and big endothelin-1 in acute myocardial-infarction. *Lancet* **2,** 53-54.

Miyauchi T. & Goto K. (1999) Heart failure and endothelin receptor antagonists. *TIPS,* **20,** 210-217.

Miyauchi T. & Masaki T. (1999) Pathophysiology of endothelin in the cardiovascular system. *Ann. Rev. Physiol.* **61,** 391-415.

Molenaar P., Oreilly G., Sharkey A. *et al.* (1993) Characterisation and localisation of endothelin receptor subtypes in the human atrio-ventricular conducting system and myocardium. *Circ. Res.* **72,** 526-538.

Molet S., Furukawa K., Maghazechi A., Hamid Q. & Giaid A. (2000) Chemokine- and cytokine-induced expression of endothelin-1 and endothelin converting enzyme-1 in endothelial cells. *J. All. Clin. Immunol.* **105,** 333-338.

Moncada S. & Higgs E.A. (1991) Endogenous nitric oxide - physiology, pathology and clinical relevance. *Eur. J. Clin. Invest.* **21,** 361-374.

Moody C.J., Dashwood M.R., Sykes R.M. *et al.* (1990) Functional and autoradiographic evidence for endothelin-1 receptors on human and rat cardiac myocytes - comparison with single smooth muscle cells. *Circ. Res.* **67,** 764-769.

Morawietz H., Talanow R., Szibor M. *et al.* (2000) Regulation of the endothelin system by shear stress in human endothelial cells. *J. Physiol.* **525,** 761-770.

Mortensen L.H. (1999) Endothelin and the central and peripheral nervous systems: A decade of endothelin research. *Clin. Exp. Pharmacol. Physiol.* **26,** 980-984.

Mulder P., Richard V., Derumeaux G. *et al.* (1997) Role of endogenous endothelin in chronic heart failure. *Circulation* **96,** 1976-1982.

Mulder P., Richard V., Bouchart F. *et al.* (1998) Selective ET_A receptor blockade prevents left ventricular remodelling and deterioration of cardiac function in experimental heart failure. *Cardiovasc. Res.* **39,** 600-608.

Mulder P., Boujedaini H., Richard V. *et al.* (2000) Selective endothelin-A versus combined endothelin-A/endothelin-B receptor blockade in rat chronic heart failure. *Circulation* **102,** 491-493.

Mullan D.M., Bell D., Kelso E.J. & McDermott B.J. (1997) Involvement of endothelin (ET)A and ETB receptors in the hypertrophic effects of ET-1 in rabbit ventricular cardiomyocytes. *J. Cardiovasc. Pharmacol.* **29,** 350-359.

Mylona P. & Cleland J.G.F. (1999) Update of REACH-1 and MERIT-HF clinical trials in heart failure. *Eur. J. Heart Failure* **1,** 197-200.

Naitoh M., Power J., Phillips P.A. *et al.* (1998) Effects of chronic AVP V_2 receptor blockade in congestive heart failure in sheep - Comparison with chronic ACE inhibition. *Adv. Exp. Med. Biol.* **449,** 445-446.

Nakahara S., Emoto N. & Matsuo M. Isolation of an alternative spliced variant of bovine ECE-2. (1999) ET-6 Conference Proceeding

Nakamura M., Arakawa N., Yoshida H. *et al.* (1993) Increased plasma endothelin concentrations in patients with acute heart failure after myocardial infarction. *Jap. Circ. J. -Eng ed.* **57,** 371-378.

Nambi P., Pullen M., Wu H.L. *et al.* (1992) Dexamethasone down-regulates the expression of endothelin receptors in vascular smooth muscle cells. *J. Biol. Chem.* **267,** 19555-19559.

Naruse M., Kawana M., Hifumi S. *et al.* (1991) Plasma-immunoreactive endothelin, but not thrombomodulin, is increased in patients with essential hypertension and ischaemic heart disease. *J. Cardiovasc. Pharmacol.* **17,** S471-S474.

Nelissen-Vrancken H.J., Debets J.J.M., Snoeckx L.H.E.H. *et al.* (1996) Time-related normalisation of maximal coronary flow in isolated perfused hearts of rats with myocardial infarction. *Circulation* **93,** 349-355.

Nicoletti A. & Michel J.B. (1999) Cardiac fibrosis and inflammation: interaction with hemodynamic and hormonal factors. *Cardiovasc. Res.* **41,** 532-543.

Nio Y., Matsubara H., Murasawa S., Kanasaki M. & Inada M. (1995) Regulation of gene-transcription of angiotensin-II receptor subtypes in myocardial infarction. *J. Clin. Invest.* **95,** 46-54.

Nitta K., Uchida K., Kimata N. *et al.* (1995) Endothelin-1 mediates erythropoietin-stimulated glomerular endothelial cell-dependent proliferation of mesangial cells. *Eur. J. Pharmacol.-Environ. Toxicol. Pharmacol. Section* **293,** 491-494.

Nolly H.L., Damiani M.T., Miatello R.M. & Abate C.D. (1997) Kallikrein, kininogen and kinins released from isolated perfused rat hearts. *Hypertension* **29,** 255.

Nunez D.J.R., Brown M.J., Davenport A.P. *et al.* (1990) Endothelin-1 messenger-RNA is widely expressed in porcine and human tissues. *J. Clin. Invest.* **85,** 1537-1541.

Ohnishi H., Oka T., Kusachi S. *et al.* (1998) Increased expression of connective tissue growth factor in the infarct zone of experimentally induced myocardial infarction in rats. *J. Mol. Cell. Cardiol.* **30,** 2411-2422.

Øie E., Vinge L.E., Tønnessen T. *et al.* (1997) Transient, isopeptide-specific induction of myocardial endothelin-1 mRNA during congestive heart failure in rats. *Am. J. Physiol. (Heart Circ. Physiol.)* **273**, H1727-H1736.

Øie E., Bjonerheim R., Grogaard H.K. *et al.* (1998) ET-receptor antagonism, myocardial gene expression, and ventricular remodelling during CHF in rats. *Am. J. Physiol. (Heart Circ. Physiol.)* **275**, H868-H877.

O'Kane S. & Ferguson M.W. (1997) Transforming growth factor betas and wound healing. *Int. J. Biochem. Cell. Biol.* **29,** A78.

Okazawa M., Shiraki T., Ninomiya H., Kobayashi S. & Masaki T. (1998) Endothelin-induced apoptosis of A375 human melanoma cells. *J. Biol. Chem.* **273,** 12584-12592.

Olivetti G., Abbi R., Quaini F. *et al.* (1997) Apoptosis in the failing human heart. *New Eng. J. Med.* **336,** 1131-1141.

Omland T., Lie R.T., Aakvaag A., Aarsland T. & Dickstein K. (1994) Plasma endothelin determination as a prognostic indicator of one-year mortality after acute myocardial infarction. *Circulation* **89,** 1573-1579.

Ono K., Eto K., Sakamoto A. *et al.* (1995) Negative chronotropic effect of endothelin-1 mediated through ET(A) Receptors in guinea-pig atria. *Circ. Res.* **76,** 284-292.

Ono K., Matsumori A., Shioi T., Furukawa Y. & Sasayama S. (1998) Cytokine gene expression after myocardial infarction in rat hearts. *Circulation* **98**, 149-156.

Opgaard O.S., Cantera L., Adner M. & Edvinsson L. (1996) Endothelin-A and -B receptors in human coronary arteries and veins. *Reg. Peptides* **63,** 149-156.

Orzechowski H.D., Richter C.M., Funke-Kaiser H. *et al.* (1997) Evidence of alternative promoters directing isoform-specific expression of human endothelin converting enzyme-1 mRNA in cultured endothelial cells. *J. Mol. Med.* **75,** 512-521.

Ozaka T., Doi Y., Kayashima K. & Fujimoto S. (1997) Weibel-Palade bodies as a storage site of calcitonin gene-related peptide and endothelin-1 in blood vessels of the rat carotid body. *Anatom. Rec.* **247,** 388-394.

Pacher R., Berglerklein J., Globits S. *et al.* (1993) Plasma big endothelin-1 concentrations in congestive heart failure patients with or without systemic hypertension. *Am. J. Cardiol.* **71,** 1293-1299.

Pacher R., Stanek B., Hulsmann M. *et al.* (1996) Prognostic impact of big endothelin-1 plasma concentrations compared with invasive hemodynamic evaluation in severe heart failure. *J. Am. Coll. Cardiol.* **27,** 633-641.

Packer M., Bristow M.R., Cohn J.N. *et al.* (1996) The effect of carvedilol on morbidity and mortality in patients with chronic heart failure. *New Eng. J. Med.* **334,** 1349-1355.

Packer M., Caspi A., Charlon V. *et al.* (1998) Multicenter, double-blind, placebo-controlled study of long-term endothelin blockade with bosentan in chronic heart failure - Results of the REACH-1 trial. *Circulation* **98,** 12.

Parameshwar J., Shackell M.M., Richardson A., Poole-Wilson P.A. & Sutton G.C. (1992) prevalence of heart failure in 3 general practices in northwest London. *Br. J. Gen. Pract.* **42,** 287-289.

Parker T.G., Packer S.E. & Schneider M.D. (1990a) Peptide growth factors can provoke fetal contractile protein gene expression in rat cardiac myocytes. *J. Clin. Invest.* **85,** 507-514.

Parker T.G., Chow K.L., Schwartz R.J. & Schneider M.D. (1990b) Modulation of cardiac and skeletal alpha-actin promoters in cardiac myocytes exhibits discordant control by TGF beta-1 and fibroblast growth factors. *Clin. Res.* **38,** A239.

Parmley W.W. (1985) Pathophysiology of congestive heart failure. *Am. J. Cardiol.* **55,** A9-A14.

Passier R.C.J.J., Smits J.F.M., Verluyten M.J.A. *et al.* (1995) Activation of angiotensin-converting enzyme expression in infarct zone following myocardial infarction. *Am. J. Physiol. (Heart Circ. Physiol.)* **38,** H1268-H1276.

Passier R.C.J.J., Smits J.F.M., Verluyten M.J.A. *et al.* (1996) Expression and localization of renin and angiotensinogen in rat heart after myocardial infarction. *Am. J. Physiol. (Heart Circ. Physiol.)* **40,** H1040-H1048.

Paul M., Bachmann J. & Ganten D. (1992) The tissue renin-angiotensin systems in cardiovascular disease. *Trends in Cardiovasc. Med.* **2,** 94-99.

Paul M., Stoll M., Kreutz R. & Fernandez-Alfonso M.S. (1996) The cellular basis of angiotensin converting enzyme mRNA expression in rat heart. *Basic Res. Cardiol.* **91,** 57-63.

Peeters A.C.T.M., Netea M.G., Kullberg B.J. *et al.* (1998) The effect of renin-angiotensin system inhibitors on pro- and anti- inflammatory cytokine production. *Immunology* **94,** 376-379.

Peiper G.M., Todd G.L., Wu S.T. *et al.* (1980) Attenuation of myocardial acidosis by propranolol during ischaemic arrest and reperfusion. *Cardiovasc. Res.* **14,** 646-653.

Peiro C., Redondo J., Rodriguez-Martinez M.A. *et al.* (1995) Influence of Endothelium on cultured vascular smooth muscle cell proliferation. *Hypertension* **25,** 748-751.

Pernow J. & Wang Q.D. (1997) Endothelin in myocardial ischaemia and reperfusion. *Cardiovasc. Res.* **33,** 518-526.

Perry G., Brown E., Thornton R. *et al.* (1997) The effect of digoxin on mortality and morbidity in patients with heart failure. *New Eng. J. Med.* **336,** 525-533.

Pfeffer M.A., Pfeffer J.M., Fishbein M.C. *et al.*. (1979) Myocardial infarct size and ventricular function in rats. *Circ. Res.* **44,** 503-512.

Pfeffer M.A., Lamas G.A., Vaughan D.E., Parisi A.F. & Braunwald E. (1988) Effect of captopril on progressive ventricular dilatation after anterior myocardial infarction. *New Eng. J. Med.* **319,** 80-86.

Pfeffer M.A. & Braunwald E. (1990) Ventricular remodelling after myocardial infarction -experimental observations and clinical implications. *Circulation* **81,** 1161-1172.

Pfeffer M.A. (1995) Left ventricular remodelling after acute myocardial infarction. *Annu.Rev.Med.* **46**, 455-466.

Piacentini L., Gray M., Honbo N.Y. *et al.* (2000) Endothelin-1 stimulates cardiac fibroblast proliferation through activation of protein kinase C. *J. Mol. Cell. Cardiol.* **32,** 565-576.

Piano M.R., Bondmass M. & Schwertz D.W. (1998) The molecular and cellular pathophysiology of heart failure. *Heart & Lung* **27,** 3-19.

Picard P., Smith P.J.W., Monge J.C. *et al.* (1998) Co-ordinated upregulation of the cardiac endothelin system in a rat model of heart failure. *J. Cardiovasc. Pharmacol.* **31,** S294-S297.

Plumpton C., Champeney R., Ashby M.J., Kuc R.E. & Davenport A.P. (1993) Characterisation of endothelin isoforms in human heart: endothelin-2 demonstrated. *J. Cardiovasc. Pharmacol.* **22,** S26-S28.

Plumpton C., Ashby M.J., Kuc R.E., Oreilly G. & Davenport A.P. (1996) Expression of endothelin peptides and mRNA in the human heart. *Clin. Sci.* **90,** 37-46.

Poole-Wilson P.A. (1989) Chronic heart failure. Causes, pathophysiology, prognosis, clinical manifestations, investigations.

Prins B.A., Hu R.M., Nazario B. *et al.* (1994) Prostaglandin E(2) and prostacyclin inhibit the production and secretion of endothelin from cultured endothelial cells. *J. Biol. Chem.* **269,** 11938-11944.

Quian S.W., Kondaiah P., Casscells W., Roberts A.B. & Spiorn M.B. (1991) A second messenger RNA species of transforming growth factor beta 1 in infarcted rat heart. *Cell Reg.* **2**, 241-249.

Rebsamen M.C., Church D.J., Morabito D., Vallotton M.B. & Lang U. (1997) Role of cAMP and calcium influx in endothelin-1-induced ANP release in rat cardiomyocytes. *Am.J.Physiol.(Endocrine Physiol.)* **273,** 922-931.

Resink T.J., Scott-Burden T., Boulanger C., Weber E. & Buhler F.R. (1990a) Internalisation of endothelin by cultured human vascular smooth muscle cells - characterisation and physiological significance. *Mol. Pharmacol.* **38,** 244-252.

Resink T.J., Hahn A.W.A., Scott-Burden T. *et al.* (1990b) Inducible endothelin messenger RNA expression and peptide secretion in cultured human vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* **168,** 1303-1310.

Rich M.W. (1997) Epidemiology, pathophysiology, and etiology of congestive heart failure in older adults. *J. Am. Geriatric. Soc.* **45,** 968-974.

Riches D.W.H. (1996) Macrophage involvement in wound repair, remodelling, and fibrosis. In: *The Molecular and Cellular Biology of Wound Repair.*, 2nd edn, pp. 95-141. Plenum Press, New York.

Rodeheffer R.J.A., Lerman D., Heublein M. & Burnett J.C.J. (1992) Increased plasma concentration of endothelin in congestive heart failure in humans. *Mayo. Clin. Proc.* **67**, 719-724.

Rosen B., Barg J. & Zimlichman R. (1999) The effects of angiotensin II, endothelin-1, and protein kinase C inhibitor on DNA synthesis and intracellular calcium mobilisation in vascular smooth muscle cells from young normotensive and spontaneously hypertensive rats. *Am. J. Hypertens.* **12,** 1243-1251.

Rossi G.P., Sacchetto A., Cesari M. & Pessina A.C. (1999) Interactions between endothelin-1 and the renin- angiotensin- aldosterone system. *Cardiovasc. Res.* **43,** 300-307.

Rubanyi G.M. & Polokoff M.A. (1994) Endothelins –molecular biology, biochemistry, pharmacology, physiology, and pathophysiology. *Pharmacol. Rev.* **46,** 325-415.

Rubanyi G.M. & Vanhoutte P.M. (2000) Hypoxia releases a vasoconstrictor substance from canine vascular endothelium. *J. Physiol.* **364,** 45-56.

Ruetten H., Thiemermann C. & Vane J.R. (1996) Endothelin-1 enhances the synthesis of tumour necrosis factor-alpha in cultured macrophages and in the anaesthetised rat. *Br. J. Pharmacol.* **117,** 31.

Ruiz-Ortega M., Gomez-Garre D., Liu X.H. *et al.* (1997) Quinapril decreases renal endothelin-1 expression and synthesis in a normotensive model of immune-complex nephritis. *J. Am. Soc. Neph.* **8,** 756-768.

Rumberger J.A. (1994) Ventricular dilatation and remodelling after myocardial-infarction. *Mayo Clin. Proceedings* **69,** 664-674.

Russell F.D., Skepper J.N. & Davenport A.P. (1998) Human endothelial cell storage granules - A novel intracellular site for isoforms of the endothelin-converting enzyme. *Circ. Res.* **83,** 314-321.

Ruwhof C. & van der Laarse A. (2000) Mechanical stress-induced cardiac hypertrophy: mechanisms and signal transduction pathways. *Cardiovasc. Res.* **47,** 23-37.

Sadoshima J. & Izumo S. (1993) Molecular characterisation of angiotensin-II-induced hypertrophy of cardiac myocytes and hyperplasia of cardiac fibroblasts - critical role of the AT(1) receptor subtype. *Circ. Res.* **73,** 413-423.

Sakai S., Miyauchi T., Sakurai Y. *et al.* (1996a) Endogenous endothelin-1 participates in the maintenance of cardiac function in rats with congestive heart failure: marked increase in endothelin-1 production in the failing heart. *Circulation* **93**, 1214-1222.

Sakai S., Miyauchi T., Kobayashi M. *et al.* (1996b) Inhibition of myocardial endothelin pathway improves long-term survival in heart failure. *Nature* **384**, 353-355.

Sakai S., Miyauchi T., Kobayashi T. *et al.* (1998) Altered expression of isoforms of myosin heavy chain mRNA in the failing rat heart is ameliorated by chronic treatment with an endothelin receptor antagonist. *J. Cardiovasc. Pharmacol.* **31**[suppl.1], S302-S305.

Sakai S., Miyauchi T. & Yamaguchi I. (2000) Long-term endothelin receptor antagonist administration improves alterations in expression of various cardiac genes in failing myocardium of rats with heart failure. *Circulation* **101,** 2849-2853.

Sakamoto A., Yanagisawa M., Sakurai T. *et al.* (1991) Cloning and functional expression of human cDNA for the ET_B endothelin receptor. *Biochem. Biophys. Res. Commun.* **178,** 656-663.

Sakurai T., Yanagisawa M., Takuwa Y. *et al.* (1990) Cloning of a cDNA-encoding a non-isopeptide-selective subtype of the endothelin receptor. *Nature* **348,** 732-735.

Sakurai T., Yanagisawa M., Inoue A. *et al.* (1991) cDNA cloning, sequence-analysis and tissue distribution of rat preproendothelin-1 messenger RNA. *Biochem. Biophys. Res. Commun.* **175,** 44-47.

Sargent C.A., Liu E.C.K., Chao C.C., Webb M.L. & Grover G.J. (1994) Role of endothelin receptor subtype B (ET_B) in myocardial ischaemia. *Life Sci.* **55,** 1833-1844.

Sawa Y., Ichikawa H., Kagisaki K. *et al.* (1998) Interleukin-6 derived from hypoxic myocytes promotes neutrophil-mediated reperfusion injury in myocardium. *J. Thorac. Cardiovasc. Surg.* **116,** 511-517.

Schaper W. & Pasyk S. (1976) Influence of collateral flow on the ischaemic tolerance of the heart following acute and subacute coronary occlusion. *Circulation* **53,** 157-162.

Schirger J.A., Hornig H., Ondrej L. & Burnett J.C. (1999) Early initiation of ET_A receptor antagonism improves cardiovascular hemodynamics in severe but not mild experimental CHF. *Circulation* **98**, A14.

Schmidt M., Kroger B., Jacob E. *et al.* (1994) Molecular characterisation of human and bovine endothelin converting enzyme (ECE-1). *Febs Lett.* **356,** 238-243.

Schrier R.W., Abdallah J.G., Weinberger H.H.D. & Abraham W.T. (2000) Therapy of heart failure. *Kid. Int.* **57,** 1418-1425.

Schunkert H., Dzau V.J., Tang S.S. *et al.* (1990) Increased rat cardiac angiotensin converting enzyme activity and messenger RNA expression in pressure overload left ventricular hypertrophy -effects on coronary resistance, contractility, and relaxation. *J. Clin. Invest.* **86,** 1913-1920.

Schunkert H., Jahn L., Izumo S., Apstein C.S. & Lovell B.H. (1991) Localisation and regulation of c-fos and c-jun protooncogene induction by systolic wall stress in normal and hypertrophied rat hearts. *P.C.N.A.* **88,** 11480-11484.

Schunkert H., Jackson B., Tang S.S. *et al.* (1993) Distribution and functional significance of cardiac angiotensin converting enzyme in hypertrophied rats. *Circulation* **87,** 1328-1339.

Schweizer A., Valdenaire O., Nelbock P. *et al.* (1997) Human endothelin-converting enzyme (ECE-1): three isoforms with distinct subcellular localisations. *Biochem. J.* **328,** 871-877.

Selye H., Bajusz E., Grasso S. & Mendell P. (1960) Simple technique for the surgical occlusion of coronary vessels in rats. *Angiology* **11,** 398-407.

Seo B., Oemar B.S., Siebenmann R. *et al.* (1994) Both ET(A) And ET(B) Receptors mediate contraction to endothelin-1 in human blood-vessels. *Circulation* **89,** 1203-1208.

Setsuta K. (1995) Origin and pathophysiological role of increased plasma endothelin-1 in patients with acute myocardial infarction. *Angiology* **46,** 557-565.

Sharif I., Kane K.A. & Wainwright C.L. (1998) Endothelin and ischaemic arrhythmias -antiarrhythmic or arrhythmogenic? *Cardiovasc. Res.* **39,** 625-632.

Shetty S.S., Okada T., Webb R.L., Delgrande D. & Lappe R.W. (1993) Functionally distinct endothelin-B receptors in vascular endothelium and smooth muscle. *Biochem. Biophys. Res. Commun.* **191,** 459-464.

Shibata S., Himeno A., Shigematsu K. *et al.* (1997) Endothelin receptors in rat pituitary gland. *Cell. Mol. Neurobiol.* **17,** 89-100.

Shimada K., Takahashi M. & Tanzawa K. (1994) Cloning and functional expression of endothelin-converting enzyme from rat endothelial cells. *J. Biol. Chem.* **269,** 18275-18278.

Shimoyama H., Sabbah H.N., Borzak S. *et al.* 1996) Short-term hemodynamic effects of endothelin receptor blockade in dogs with chronic heart failure. *Circulation* **94,** 779-784.

Shubeita H.E., Mcdonough P.M. *et al.* (1990) Endothelin induction of inositol phospholipid hydrolysis, sarcomere assembly, and cardiac gene expression in ventricular myocytes -a paracrine mechanism for myocardial cell hypertrophy. *J. Biol. Chem.* **265,** 20555-20562.

Singer R.H., Lawerence J.B. & Villnave C. (1986) Optimisation of *in situ* hybridisation using isotopic and non-isotopic detection methods. *Biotechniques* **4,** 230.

Smith P.J.W., Ornatsky O., Stewart D.J. *et al.* (2000) The effects of estrogen-replacement on infarct size, cardiac remodelling, and the endothelin system following myocardial infarction in ovariectomised rats. *Circulation* In Press.

Smith W.M. (1985) Epidemiology of congestive heart failure. *Am. J. Cardiol.* **55,** A3-A8

Smits J.F.M., van Krimpen C., Schoemaker R.G. *et al.* (1992) Angiotensin II receptor blockade after myocardial infarction in rats: effects on hemodynamics, myocardial DNA synthesis, and interstitial collagen content. *J. Cardiovasc. Pharmacol.* **20**, 772-778.

Sokolovsky M. (1994) Endothelins and sarafotoxins -receptor heterogeneity. *Int. J. Biochem.* **26,** 335-340.

Spratt J.C.S., Goddard J., Labinjoh C. *et al.* (1999) The haemodynamic effects of systemic endothelin A receptor antagonism in healthy humans in vivo. *Br. J. Clin. Pharmacol.* **47,** 576.

Stein B., Frank P., Schmitz W., Scholz H. & Thoenes M. (1996) Endotoxin and cytokines induce direct cardiodepressive effects in mammalian cardiomyocytes via induction of nitric oxide synthase. *J. Mol. Cell. Cardiol.* **28,** 1631-1639.

Stewart D.J., Kubac G., Costello K.B. & Cernacek P. (1991) Increased plasma endothelin-1 in the early hours of acute myocardial infarction. *J. Am. Coll. Cardiol.* **18,** 38-43.

Stewart D.J., Teichert-Kuliszewska K., Bendeck M.P. *et al.* (1996) Expression of nitric oxide synthase III and endothelin-1 in an *in vitro* model of angiogenesis. *Circulation* **94,** 895.

Strachan F.E., Spratt J.C., Wilkinson I.B. *et al.* (1999) Systemic blockade of the endothelin-B receptor increases peripheral vascular resistance in healthy men. *Hypertension* **33,** 581-585.

Sudjarwo S.A., Hori M., Tanaka T. *et al.* (1994) Subtypes of endothelin ET(A) and ET(B) receptors mediating venous smooth muscle contraction. *Biochem. Biophys. Res. Commun.* **200,** 627-633.

Sugden P.H. & Bogoyevitch M.A. (1996) Endothelin-1-dependent signalling pathways in the myocardium. *Trends in Cardiovasc. Med.* **6,** 87-94.

Sumner M.J., Cannon T.R., Mundin J.W., White D.G. & Watts I.S. (1992) Endothelin ET_A and endothelin ET_B receptors mediate vascular smooth muscle contraction. *Br. J. Pharmacol.* **107,** 858-860.

Sun Y.O. & Ramires F.J.A. (1996) Transforming growth factor-beta 1 (TGF-beta 1) receptor binding in the infarcted rat heart. *Circulation* **94,** 747.

Sun Y.O., Zhang J.Q., Zhang J.K. *et al.* (1998) Angiotensin II, transforming growth factor-beta(1) and repair in the infarcted heart. *J. Mol. Cell. Cardiol.* **30,** 1559-1569.

Sun Y.O. & Weber K.T. (2000) Infarct scar: a dynamic tissue. *Cardiovasc. Res.* **46,** 250-256.

Sun Y.O., Zhang J.Q., Zhang J. *et al.* (2000) Cardiac remodelling by fibrous tissue after infarction in rats. *J. Lab. Clin. Med.* **135,** 316-323.

Sunako M., Kawahara Y. & Kariya K. (1989) Endothelin-induced biphasic formation of 1,2-diacylglycerol in cultured rabbit vascular smooth muscle cells - mass analysis with a radio-enzymatic assay. *Biochem. Biophys. Res. Commun.* **160,** 744-750.

Suzuki T., Kumazaki T., & Mitsui Y. (1993) Endothelin-1 is produced and secreted by neonatal rat cardiac myocytes in vitro. *Biochem. Biophys. Res. Commun.* **191,** 823-830.

Swan H.J.C. (1993) Left ventricular systolic and diastolic dysfunction in the acute phases of myocardial ischaemia and infarction, and in the later phases of recovery. *Eur. Heart J.* **14,** SA48-SA56.

Swedberg K. (1987) Effects of enalapril on mortality in severe congestive-heart-failure -results of the co-operative north scandinavian enalapril survival study (CONSENSUS). *New Eng. J. Med.* **316,** 1429-1435.

Swynghedauw B. (1999) Molecular mechanisms of myocardial remodelling. *Physiol. Rev.s* **79,** 215-262.

Takahashi M., Matsushita Y., Iijima Y. & Tanzawa K. (1993) Purification and characterisation of endothelin-converting enzyme from rat lung. *J. Biol. Chem.* **268,** 21394-21398.

Takahashi N., Calderone A., Izzo J.N.J. *et al.* (1994) Hypertrophic stimuli induce transforming growth factor beta-1 expression in rat ventricular myocytes. *J.Clin.Invest.* **94,** 1470-1476.

Takanashi M. & Endoh M. (1991) Characterization of positive inotropic effect of endothelin on mammalian ventricular myocardium. *Am. J. Physiol.* **261,** H611-H619.

Takemura G., Ohno M., Hayakawa Y. *et al.* (1998) Role of apoptosis in the disappearance of infiltrated and proliferated interstitial cells after myocardial infarction. *Circ. Res.* **82,** 1130-1138.

Takuwa N., Takuwa Y., Yanagisawa M., Yamashita K. & Masaki T. (1989) A novel vasoactive peptide endothelin stimulates mitogenesis through inositol lipid turnover in swiss 3T3 fibroblasts. *J. Biol. Chem.* **264,** 7856-7861.

Tamura T., Said S., Harris J., Lu W. & Gerdes A.M. (2000) Reverse remodelling of cardiac myocyte hypertrophy in hypertension and failure by targeting of the renin-angiotensin system. *Circulation* **102,** 253-259.

Teerlink J.R., Loffler B.M., Hess P., Maire J.P., Clozel M. & Clozel J.P. (1994) Role of endothelin in the maintenance of blood pressure in conscious rats with chronic heart failure -acute effects of the endothelin receptor antagonist Ro-47-0203 (bosentan). *Circulation* **90,** 2510-2518.

Tharaux P.L., Chatziantoniou C., Casellas D. *et al.* (1999) Vascular endothelin-1 gene expression and synthesis and effect on renal type I collagen synthesis and nephroangiosclerosis during nitric oxide synthase inhibition in rats. *Circulation* **99,** 2185-2191.

Thompson N.L., Bazoberry F., Speir E. *et al.* (1988) Transforming growth factor beta-1 in acute myocardial infarction in fats. *Growth Fac.* **1,** 91-99.

Timms A.D. & Davies S.W. (2000) In: *Heart Failure,* Grower Medical Publishing.

Tomita H., Egashira K., Ohara Y. *et al.* (1998) Early induction of transforming growth factor-beta via angiotensin II type 1 receptors contributes to cardiac fibrosis induced by long-term blockade of nitric oxide synthesis in rats. *Hypertension* **32,** 273-279.

Tomita K., Nonoguchi H. & Marumo F. (1990) Effects of endothelin on peptide-dependent cyclic adenosine monophosphate accumulation along the nephron segments of the rat. *J. Clin. Invest.* **85,** 2014-2018.

Tomita K., Nonoguchi H., Terada Y. & Marumo F. (1993) Effects of ET-1 on water and chloride transport in cortical collecting ducts of the rat. *Am. J. Physiol.* **264,** F690-F696.

Tønnessen T., Giaid A., Yanagasawia M. *et al.* (1993) Release of endothelin from the porcine heart after short-term coronary artery occlusion. *Cardiovasc. Res.* **27,** 1482-1485.

Tønnessen T., Giaid A., Yanagasawia M. & Christensen G. (1995) Increased *in vivo* expression and production of endothelin-1 by porcine cardiomyocytes subjected to ischaemia. *Circ. Res.***76,** 767-772.

Tønnessen T., Christensen G., Oie E..*et al.*. (1997) Increased cardiac expression of endothelin-1 mRNA in ischaemic heart failure in rats. *Cardiovasc. Res.* **33,** 601-610.

Tønnessen T., Lunde P.K., Giaid A. *et al.* (1998) Pulmonary and cardiac expression of preproendothelin-1 mRNA are increased in heart failure after myocardial infarction in rats. Localisation of preproendothelin-1 mRNA and endothelin peptide. *Cardiovasc. Res.* **39**, 633-643.

Trocha S.D., Jones S.P., Kevil C.G. *et al.* (1999) Adhesion molecule deficient mice and myocardial ischaemia-reperfusion injury. *Circulation* **100,** A2869.

Turner A.J. & Murphy L.J. (1995) Molecular pharmacology of endothelin converting enzymes. *Biochem. Pharmacol.* **51,** 91-102.

Tzanidis A. & Lim S. (1999) Does endothelin mediate cardiac fibrosis following myocardial infarction via activation of transforming growth factor beta 1? *Circulation* **100,** A279.

Uddman E., Moller S., Adner M. & Edvinsson L. (1999) Cytokines induce increased endothelin ET_B receptor-mediated contraction. *Eur. J. Pharmacol.* **376,** 223-232.

Unger T., Culman J. & Gohlke P. (1998) Angiotensin II receptor blockade and end-organ protection: pharmacological rationale and evidence. *J. Hypert.* **16,** S3-S9.

Urata H., Kinoshita A., Perez D.M. *et al.* (1991) Cloning of the gene and cDNA for human heart chymase. *J. Biol. Chem.* **266,** 17173-17179.

Valdenaire O., Rohrbacher E. & Mattei M.G. (1995) Organisation of the gene encoding the human endothelin-converting enzyme (ECE-1). *J. Biol. Chem.* **270,** 29794-29798.

Valdenaire O., Lepailleur-Enouf D., Egidy G. *et al.* (1999) A fourth isoform of endothelin-converting enzyme (ECE-1) is generated from an additional promoter - Molecular cloning and characterisation. *Eur. J. Biochem.* **264,** 341-349.

Velasco C.E., Yanagisawa M., Williamson J.L. & Triana J.F. (1993) Cardioprotective action of endothelin-1 during ischemia. *Circulation* **88,** 544.

Vigne P., Marsault R., Breittmayer J.P. & Frelin C. (1990) Endothelin stimulates phosphatidylinositol hydrolysis and DNA -synthesis in brain capillary endothelial cells. *Biochem. J.* **266,** 415-420.

Vila-Petroff M.G., Younes A., Egan J., Lakatta E.G. & Sollott S.J. (1999) Activation of distinct cAMP-dependent and cGMP-dependent pathways by nitric oxide in cardiac myocytes. *Circ. Res.* **84,** 1020-1031.

Villaschi S. & Nicosia R.F. (1994) Paracrine interactions between fibroblasts and endothelial cells in a serum-free coculture model -modulation of angiogenesis and collagen gel contraction. *Lab. Invest.* **71,** 291-299.

Vitola J.V., Forman M.B., Holsinger J.P. *et al.* (1996) Role of endothelin in a rabbit model of acute myocardial infarction: Effects of receptor antagonists. *J. Cardiovasc. Pharmacol.* **28,** 774-783.

Vonlutterotti N., Catanzaro D.F., Sealey J.E. & Laragh J.H. (1994) Renin is not synthesised by cardiac and extrarenal vascular tissues - a review of experimental evidence. *Circulation* **89,** 458-470.

Vracko R., Thorning D. & Frederickson R.G. (1989) Connective tissue cells in healing rat myocardium -a study of cell reactions in rhythmically contracting environment. *Am. J. Pathol.* **134,** 993-1006.

Wahl G.M., Stern M. & Stark G.R. (1979) Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymtehyl paper and rapid hybridisation by using dextran sulfate. *Proc. Natl. Acad. Sci.* **76,** 3683-3687.

Wahl S.M., McCartney-Francis N., Allen J.B. *et al.* (1990) Macrophage production of TGF-beta and regulation by TGF-beta. *Ann. New York Acc. Sci.* **593,** 188-196.

Wang D.L., Chen J.J., Shin N.L. *et al.* (1992) Endothelin stimulates cardiac alpha-myosin and beta-myosin heavy chain gene expression. *Biochem. Biophys. Res. Commun.* **183,** 1260-1265.

Wang Q.D., Li X.S. & Pernow J. (1994) Characterisation of endothelin-1-induced vascular effects in the rat heart by using endothelin receptor antagonists. *Eur. J. Pharmacol.* **271,** 25-30.

Wang Q.D., Hemsen A., Li X.S. *et al.* (1995) Local overflow and enhanced tissue content of endothelin following myocardial ischaemia and reperfusion in the pig - modulation by L- arginine. *Cardiovasc. Res.* **29,** 44-49.

Wang Y.Z., Pouyssegur J. & Dunn M.J. (1994) Endothelin stimulates mitogen-activated protein kinase activity in mesangial cells through ET(A). *J. Am. Soc. Neph.* **5,** 1074-1080.

Warford A. (1988) *In situ* hybridisation -a new tool in pathology. *Med. Lab. Sci.* **45,** 381-394.

Watanabe T., Suzuki N., Shimamoto N., Fujino M. & Imada A. (1991) Contribution of endogenous endothelin to the extension of myocardial infarct size in rats. *Circ. Res.* **69,** 370-377.

Weber K.T., Janicki J.S., Shroff S.G. *et al.* (1988) Collagen remodelling of the pressure overloaded, hypertrophied non-human primate myocardium. *Circ. Res.* **62,** 757-765.

Weber K.T. & Brilla C.G. (1991) Pathological hypertrophy and cardiac interstitium - fibrosis and renin-angiotensin-aldosterone system. *Circulation* **83,** 1849-1865.

Weber K.T., Sun Y., Tyagi S.C. & Cleutjens J.P.M. (1994) Collagen network of the myocardium: Function, structural remodelling and regulatory mechanisms. *J. Mol. Cell. Cardiol.* **26,** 279-292.

Weber K.T. (1997) Extracellular matrix remodelling in heart failure -A role for *de novo* angiotensin II generation. *Circulation* **96,** 4065-4082.

Wei C.M., Heublein D.M., Perrella M.A. *et al.* (1993) Natriuretic peptide system in human heart failure. *Circulation* **88,** 1004-1009.

Wei C.M., Lerman A., Rodeheffer R.J. *et al.* (1994) Endothelin in human congestive heart failure. *Circulation* **89**, 1580-1586.

Weissberg P.L., Witchell C., Davenport A.P., Hesketh T.R. & Metcalfe J.C. (1990) The endothelin peptides ET-1, ET-2, ET-3 and sarafotoxin S6b are co-mitogenic with platelet-derived growth factor for vascular smooth muscle cells. *Atherosclerosis* **85,** 257-262.

Wharton J., Rutherford R.A.D., Gordon L. *et al.* (1991) Localisation of endothelin binding sites and endothelin-like immunoreactivity in human fetal heart. *J. Cardiovasc. Pharmacol.* **17,** S378-S384.

Whittaker P. (1997) Collagen and ventricular remodelling after acute myocardial infarction: Concepts and hypotheses. *Basic Res. Cardiol.* **92,** 79-81.

Wieczorek I., Haynes W.G., Webb D.J. *et al.* (1994) Raised plasma endothelin in unstable angina and non-q-wave myocardial infarction -relation to cardiovascular outcome. *Br. Heart J.* **72,** 436-441.

Willingham M.C. (1999) Cytochemical methods for the detection of apoptosis. *J. Histochem. Cytochem.* **47,** 1101-1109.

Wollert K.C. & Drexler H. (1999) The renin-angiotensin system and experimental heart failure. *Cardiovasc. Res.* **43,** 838-849.

Xu D., Emoto N., Giaid A., Slaughter C., Kaw S., Dewit D. & Yanagisawa M. (1994) ECE-1 -a membrane-bound metalloprotease that catalyses the proteolytic activation of big endothelin-1. *Cell* **78,** 473-485.

Yamazaki T., Kudoh S., Zou Y.Z. *et al.* (1995) Endothelin-1 and angiotensin II synergistically mediate stretch-induced cardiac hypertrophy. *Circulation* **90,** [suppl I], A569.

Yanagisawa H., Yanagisawa M. *et al.* (1998) Dual genetic pathways of endothelin-mediated intercellular signalling revealed by targeted disruption of endothelin converting enzyme-1 gene. *Development* **125,** 825-836.

Yanagisawa M., Kurihara H., Kimura S. *et al.* (1988) A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* **332,** 411-415.

Yaoita H., Ogawa K., Maehara K. & Maruyama Y. (2000) Apoptosis in relevant clinical situations: contribution of apoptosis in myocardial infarction. *Cardiovasc. Res.* **45,** 630-641.

Yeh Y.C., Burns E.R., Yeh J. & Yeh H.W. (1991) Synergistic effects of endothelin-1 (ET-1) and transforming growth factor-alpha (TGF-alpha) or epidermal growth factor (EGF) on DNA replication and G_1 to S-phase transition. *Biosci. Reports* **11,** 171-180.

Yokokawa K., Tahara H., Kohno M. *et al.* (1993) Heparin regulates endothelin production through Endothelium-derived nitric oxide in human endothelial cells. *J. Clin. Invest.* **92,** 2080-2085.

Yorikane R., Sakai S., Miyauchi T. *et al.* (1993) Increased production of endothelin-1 in the hypertrophied rat heart due to pressure overload. *Febs Lett.* **332,** 31-34.

Zeidel ML., Brady HR., Kone B., Gullans SR. & Brenner BM. (1989) Endothelin, a peptide inhibitor of Na^+/K^+ ATPase in intact tubular epithelial cells. *Am.J.Physiol.(Heart Circ.Physiol)* **257,** C1101-C1107.

Zhang Y., Oliver J.R., & Horowitz J.D. (1998) Endothelin B receptor-mediated vasoconstriction induced by endothelin A receptor antagonist. *Cardiovasc. Res.* **39,** 665-673.

Ziche M., Morbidelli L., Donnini S. *et al.* (1995) ET(B) receptors promote proliferation and migration of endothelial cells. *J. Cardiovasc. Pharmacol.* **26,** S284-S286.

Zile M.R., Tomita M., Ishihara K. *et al.* (1993) Changes in diastolic function during development and correction of chronic LV volume overload produced by mitral regurgitation. *Circulation* **87,** 1378-1388.

Zouki C., Baron C., Fournier A. *et al.* (1999) Endothelin-1 enhances neutrophil adhesion to human coronary artery endothelial cells: role of ET_A receptors and platelet-activating factor. *Br. J. Pharmacol.* **127,** 969-979.